THE USE OF PHYSIOLOGICAL INDICATOR TRAITS AS JUVENILE PREDICTORS IN DAIRY CATTLE

Thesis submitted in accordance with the requirements of the
University of Liverpool for the degree of Doctor in Philosophy
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To Mum and Dad

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ABSTRACT

Average fertility levels in the UK dairy population are low (pregnancy rate to first service 39.7%; Royal *et al.*, 2000a; conception rate to first service 37%; Mayne *et al.*, 2002) and indeed similar around the world. Intense selection for milk yield over the last 30 years is, in part, responsible for the genetic and phenotypic decline in fertility. Many countries, including the UK, now publish and use genetic evaluations for female fertility (Miglior *et al.*, 2005). An addition to fertility indices of an appropriate indicator trait for female fertility which is measurable in the juvenile male could increase the rate of genetic improvement in fertility. Subsequent to work by Land (1973) who first proposed that sex linked characters in the female are expressed in the male, several studies in calves and lambs have looked at potential physiological juvenile indicator traits for female reproduction (e.g. Haley *et al.*, 1989; Haley *et al.*, 1990; Mackinnon *et al.*, 1991; Royal *et al.*, 2000). These studies have focused on reproductive hormones (testosterone, luteinizing hormone), however, to date and to the author's knowledge, no studies have investigated the potential of metabolic hormones as juvenile predictors at a genetic level.

The length and severity of negative energy balance postpartum is unfavourably correlated (genetically & phenotypically) with interval to first ovulation (de Vries & Veerkamp, 2000). During this period concentrations of free fatty acids (FFA), glucose, growth hormone (GH), insulin, insulin like growth factor 1 (IGF-1) and other hormones, all of which have links with many aspects of reproduction, are altered. A moderate heritability and genetic correlation between these parameters in the female and male calf with female fertility could allow juvenile selection for fertility. With this in mind the aims of this thesis were to estimate the heritability of FFA, glucose, GH, insulin and IGF-1 in male and female calves and to assess the genetic and phenotypic relationships of these with production and fertility traits.

Three datasets were utilised for this thesis; datasets D1 and D2 were collected using UK dairy calves whilst D3 was collected as part of a Danish study (Chapter 3). In the first study (Chapter 4) FFA, glucose, GH and insulin measurements in 9 month old male Danish dairy calves (D3, Chapter 3; Danish Holstein n = 1047, Danish Jersey n = 200 and Red Dane n = 251) following overnight fast were analyzed. Fertility estimated breeding values (FertEBV) were available for a subset (n = 810) of the male calves as adult sires (Danish Cattle Federation, Aarhus). This study found that a considerable amount of the phenotypic variance in FFA, glucose and insulin appears to be genetic in male 9 month old Danish dairy calves. The approximate genetic correlations between FertEBV with FFA and glucose were negative and significant. Therefore, on average, male calves with high glucose and FFA following an overnight fast at 9 months of age tend to produce female offspring with reduced fertility.

In the second study (Chapter 5 & 6) FFA, glucose, GH, insulin and IGF-1 measurements in fed 4-5 month old male and female dairy calves (D1-F, n = 326 females; D1-M, n = 256 males; D2-F, n = 496 females; Chapter 3) were analysed. Furthermore, fertility measures: interval to commencement of luteal activity postpartum (CLA) and the proportion of milk samples with luteal activity upto 60 days postpartum when sampling is three times a week (PLA_a), weekly (PLA_w), fortnightly (PLA_f) or monthly (PLA_m) were calculated for a subset of the females in D1 and D2 (n = 440). Results showed that FFA, glucose, GH, insulin and IGF-1 were all moderately heritable in male and female calves (range 0.09 ± 0.05 to 0.66 ± 0.14; Chapter 5). Furthermore, the five hormones and metabolites showed moderate genetic correlations with one another and with weight. Genetic and phenotypic correlations between FFA, glucose, GH, insulin and IGF-1 with weight were positive and moderate. This indicates that at a genetic level it is possible that certain alleles influence both weight and the hormone or metabolite concentration, i.e. pleiotropy, or that genes controlling weight and the hormone or metabolite are positioned close to one another and therefore often inherited together, i.e. linkage disequilibrium, and at a phenotypic level higher weight animals tend to have higher concentrations of the above than lighter animals. Glucose, insulin and IGF-1 were all

positively genetically correlated with each other whereas glucose and FFA were negatively genetically correlated.

The genetic and phenotypic relationships between the hormones and metabolites with physiological fertility parameters were all non-significant. Of interest though was that glucose and insulin were moderately, negatively genetically correlated with CLA indicating that fed calves with high glucose and insulin concentrations at 4 months of age then proceed to have a shorter CLA as heifers. Also of interest was the moderate and negative genetic correlation between glucose and FFA with PLA_a and PLA_w suggesting that low concentration of glucose and FFA in 4 month old calves is genetically related to a high proportion of milk samples with luteal activity during the first 60 days postpartum.

The regression of the hormone and metabolites on sire PTAs (Chapter 6) found significant negative approximate genetic correlations between the fertility index and GH, and a positive significant genetic correlation between GH and calving interval. These perhaps indicate that calves with a good ability to secrete GH at 4 months of age proceed to have high GH concentration postpartum and perhaps some of the problems associated with NEB i.e. poor fertility (Butler, 2000; de Vries & Veerkamp, 2000; Dechow *et al.*, 2002). The regressions also found a significant negative association between milk protein percentage with FFA whilst a positive association between milk protein percentage with insulin. These are reflective of the concentrations of insulin and FFA in different energy states. Insulin is high when glucose is high and therefore substrates are available to produce milk proteins whereas FFA concentrations are high when lipolysis is occurring during energy deficit and therefore milk protein production is limited.

In summary the results of this thesis have provided evidence to show that FFA, glucose, GH, insulin and IGF-1 are heritable in UK and Danish male and female calves (4 and 9 months of age). In the Danish experiment (Chapter 4), male calves with low FFA and glucose concentrations at 9 months of age following a fast were more likely to produce female offspring with a better fertility index than those with high FFA and glucose

concentrations. Yet in the UK experiment (Chapter 5 & 6), high glucose and insulin concentrations in fed calves at 4 months of age were genetically more likely to have a shorter interval to luteal activity as heifers. The conflicting relationship with glucose may be due to the effect of fasting. Perhaps a calf with high glucose concentrations during normal feeding that is able to quickly reduce its glucose during a fast then goes on to have better fertility. It would appear that an animals' ability to withstand metabolic stress postpartum, in the form of negative energy balance, is reflected in hormone and metabolite concentrations as a calf. The few inconsistencies in the results are likely due to differences between fed and fasted calves, few records for CLA and PLA fertility measures and PLA being difficult to interpret. Further analysis with a larger dataset is needed to confirm the findings here before the use of glucose, FFA and insulin concentrations in the calf as juvenile predictor traits for fertility could be considered.

DECLARATION

I hereby declare that the studies undertaken herein, except where due acknowledgement is made by reference, were the unaided work of the author. No part of this work has been previously submitted by the candidate for another degree. I also acknowledge all assistance given to me during my preparation of this thesis.

Catherine Hayhurst

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AI	Artificial insemination
AI-REML	Average information restricted maximum likelihood method
ALL	All breeds combined (Danish Holstein, Danish Jersey and Red Dane)
ATP	Adenosine triphosphate
BCS	Body condition score
CLA	Interval to commencement of luteal activity post-partum
COV	Covariance
D1	Dataset 1
D1-M	Dataset 1 males
D1-F	Dataset 1 females
D2	Dataset 2
D3	Dataset 3
DH	Danish Holstein
DOVI	Delayed ovulation type I
DOVII	Delayed ovulation type II
DTPA	Diethylene triamine penta acetic acid
EBV	Estimated breeding value
ED	Estimated dose
ELISA	Enzyme linked immuno-sorbent assay
FertEBV	Danish estimated breeding values for female fertility
FFA	Free fatty acids
FI	Fertility index
FSH	Follicle stimulating hormone
GAS	Gene assisted selection
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GnRH	Gonadotrophin releasing hormone
h^2	Heritability

IFNτ	Interferon Tau
IGF-1	Insulin like growth factor one
IGF-1R	IGF-1 receptors
IGFBP	Insulin like growth factor binding protein
InLut	Length of inter-luteal phase
InOvul	Length of inter-ovulatory interval
IVEP	In vitro embryo production
LH	Luteinising hormone
L-NAME	N-G-nitro-L-arginine methyl ester
LUT	Length of luteal phase
MAS	Marker assisted selection
MDC	Milk development council
MOET	Multiple ovulation and embryo transfer
NAD	Nicotinamide adenine dinucleotide
NEB	Negative energy balance
NMA	N-methyl-D,L-aspartic acid
NMR	National milk records
NR56	Non return to oestrus rate 56 days after artificial insemination
NS	Not significant ($P>0.05$, unless otherwise stated)
PCLI	Persistent corpus luteum type I with delayed luteolysis during the first cycle
PCLII	Persistent corpus luteum type II with delayed luteolysis during subsequent cycles before insemination
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PIF	Prolactin inhibiting factor
PIN	Production index
PLA	Percentage of milk samples with luteal activity upto 60 days postpartum
PLA_a	Percentage of milk samples with luteal activity upto 60 days postpartum using all samples collected

PLA_w	Percentage of milk samples with luteal activity upto 60 days postpartum using weekly milk samples
PLA_f	Percentage of milk samples with luteal activity upto 60 days postpartum using fortnightly milk samples
PLA_m	Percentage of milk samples with luteal activity upto 60 days postpartum using monthly milk samples
PLI	Profitable lifetime index
PTA	Predicted transmitting ability
r_A	Genetic correlation
r_P	Phenotypic correlation
r_E	Environmental correlation
rbGH	Recombinant bovine growth hormone
REML	Restricted maximum likelihood method
RIA	Radioimmunoassay
SCC	Somatic cell count
S.D	Standard deviation
S.E	Standard error of the mean
TOP	Total overall profit
TRH	Thyrotrophin releasing hormone
TSH	Thyroid stimulating hormone
TGF-β	Transforming growth factor-β

Chapter 1: LITERATURE REVIEW

In this chapter areas of animal physiology and genetic methodology will be reviewed. The research described in this thesis (Chapters 2-7) involves the genetic analyses of data collected on juvenile male and female calves (4-5 and 9 months of age) from Danish and UK dairy cattle populations. Additionally, adult female cows were studied with respect to their fertility and milk production postpartum. Therefore in this review of literature subjects covered will include: UK and Danish dairy breeding industries, reproductive physiology and endocrinology in the juvenile male, juvenile female and mature female, genetics of fertility and methods used to estimate genetic parameters. Furthermore, the decline in female fertility and fertility improvement programs will be described. Finally, previous work on juvenile predictors will be discussed, in addition to potential hormones and metabolites that could be studied.

1.1 INTRODUCTION

Domestic cattle belong to the family *Bovidae*. The family can be divided into *Bos taurus* (temperate, European) and *Bos indicus* (tropical, Zebu). Both genotypes originate from the same breed in 4500BC in Western Asia yet differ greatly in many aspects. The domestic cow is polyoestrous from puberty and has consecutive oestrous cycles lasting approximately 21 days with ovulation occurring on day 0/1 of the cycle. The modern dairy cow, the most abundant breed being the Holstein, has been intensely selected for milk yield with little consideration to functional traits until recently (Miglior *et al.*, 2005). This has led to dairy breeds which can produce a great deal of milk, however, health and fertility are becoming serious concerns to most dairy farmers.

1.2 DAIRY INDUSTRY

The following describes the UK and the Danish dairy industry, although practices are similar in other countries.

1.2.1 The UK dairy industry

The UK dairy industry in 2003 had approximately 2,191,000 dairy cows (54% Holstein Friesian, 31% Friesian, 7% Holstein, and 8% other) with an average herd size of 92 (Milk Development Council, Trent Lodge, Stroud Road, Cirencester, Gloucestershire).

Advancements in techniques used over the last 50 years, e.g. development of artificial insemination (AI), semen freezing, multiple ovulation and embryo transplantation (MOET) and semen sexing, have increased the rate of genetic improvement possible in the dairy breeding industry. There are now many different breeding schemes used by breeding companies to achieve the best progress in their bull and cow breeding.

1.2.1.1 Breeding schemes

The structure of breeding schemes is essentially the same in most breeding companies (*Figure 1.1*). Females are bred to become either dams of bulls or cows and males are bred to become either sires of cows or bulls, leading to four different breeding goals.

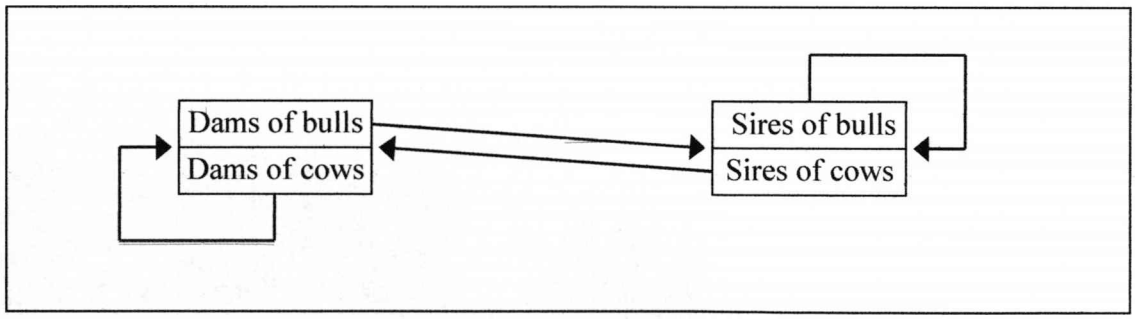

Figure 1.1 Representation of the four pathways of selection used in dairy breeding companies.

1.2.1.1.1 Progeny test schemes

After the use of AI became popular, progeny testing was implemented in dairy breeding companies and is now used in most developed countries. Progeny testing involves the

comparison of bulls based on the performance, in milk yield and other traits, of his daughters (Simm, 2000a). At puberty, between 9-12 months of age (Evans *et al.*, 1996), a young sire will undergo semen collection to produce enough semen for ≥100 AI straws and these straws are distributed to farms (at a low price as unproven young sires). One year later, a number of daughters of the young sire are born. At approximately 12 months of age the daughters are inseminated. Therefore, when the young sire is 4-5 years of age his daughters will be lactating and performance data can be recorded by the milk recording company. At the earliest, a young sire will be 5 years of age when its first breeding values (proofs) are obtained (Simm, 2000a; *Figure 1.2*).

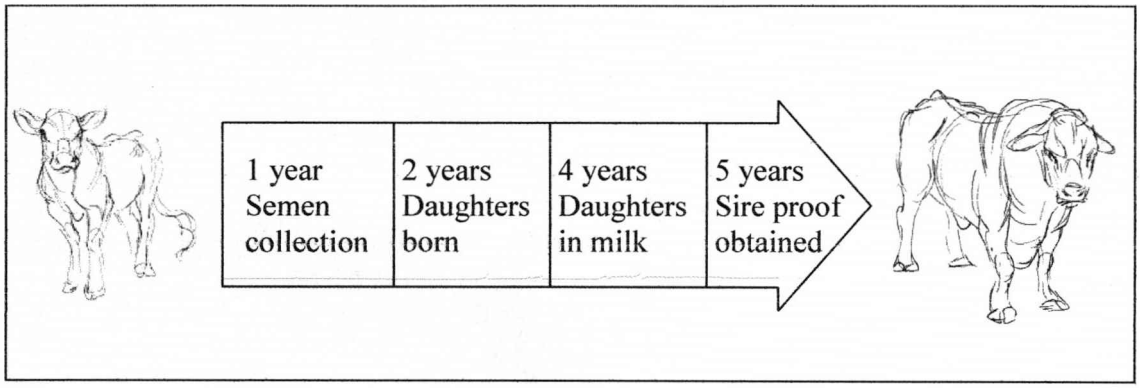

Figure 1.2 Schematic representation of time taken from birth until a proof is obtained for a young sire.

The advantage of progeny test schemes is the high reliability of the proofs obtained which increases with further lactation records and as the number of daughters increases. The limitation of traditional progeny test schemes is the long generation interval and low reproductive rate in females (Dekkers, 1992). The long generation interval is partly due to the requirement of at least one lactation record for females before they are selected to become dams of bulls (Dekkers, 1992). Sires must also have a progeny test before being used to sire prospective bulls, which means waiting until they are 5 years old (Dekkers, 1992). Traditional progeny test schemes, with a restriction of one complete lactation for dams of bulls and one progeny test for sires of bulls can result in genetic gain of approximately 0.109 genetic standard deviations per year (Dekkers, 1992).

1.2.1.1.2 Nucleus scheme with MOET

Nucleus herds were initiated for several reasons including: to reduce costs, increase the number of traits recorded and increase the accuracy of measurements involved in progeny testing. Nucleus herds are used by breeding companies to test their own bull dams in a commercial environment and often selection decisions are made before progeny test results thus reducing the generation interval leading to greater rates of genetic gain (Dekkers, 1992). Nucleus herds can be either closed or open and this applies to both the males and females. Open herds will obtain replacement heifers, embryos, semen and bulls when needed. Closed herds will receive no replacements from external sources. However, closed herds will need external inputs of genetics periodically due to the inevitable reduction of genetic variation caused by the population being closed (Dekkers, 1992).

Greater genetic progress has been achieved since the introduction of MOET in many breeding companies. MOET involves hormonal treatment of a female of high genetic merit to induce multiple ovulations. The female is then inseminated, multiple embryos are removed after seven days and implanted into recipient females or frozen and used at a later stage. MOET allows many offspring to be produced by cows of high genetic merit. Another benefit of MOET is that many full sibs can be produced. A young bull can be selected by assessing the performance of his full sib sisters during their first lactation. This is less accurate than a full progeny test but reduces the generation interval. The selection of an elite female to superovulate can be made after one lactation based on production and conformation data or at puberty based on pedigree information. The latter is less accurate but generation interval and costs of rearing are reduced.

1.2.1.2 Selection indices

In the UK genetic evaluations, calculated by MDC Evaluations (Milk Development Council) display breeding values for individual traits and values for the common selection indices used (*Table 1.1*).

Table 1.1 Example of the genetic evaluations produced for bulls in the UK (MDC Evaluations).

Rank	Res. Ind	Bull Name	Breed	Bull HBN	Dtrs	Hrds	%dtr top	% top 2	Lacts	Rel %
1	ITB	XXX	1	XXX	192	127	4	2	321	97
2	ITB	XXX	1	XXX	49	32	10	8	49	88
3	ITB	XXX	1	XXX	849	342	3	2	1041	99
4	COM	XXX	65	XXX	68	25	19	16	106	94
5	ITB	XXX	65	XXX	310	66	6	6	329	98

Milk (kg)	Fat (kg)	Prot (kg)	Fat (%)	Prot (%)	SCC Rel	SCC	LS Rel	LS
595	26.7	17.8	0.05	-0.02	85	-26	70	0.5
764	30.2	25.5	0.01	0.01	46	3	29	0
708	17.7	25	-0.11	0.03	91	-15	79	0.2
637	27.2	19.9	0.04	-0.01	87	-14	77	0.2
743	30.8	21.7	0.03	-0.03	97	-6	81	0.1

FI Rel	FI	Pers.	Type	£PIN	£PLI	Available from	Available NI
74	-1.6	59	3.2	57	82	GEN	
49	-5.7	58	1.9	77	77	COG	
83	-1.8	59	1.3	64	75	GEN	
61	-0.4	54	0.6	62	72	SW	
69	-1.3	54	-0.3	67	70	INI	AIS

Additional important information

Res.Ind (Results indicator) - shows the type of evaluation the bull has (UK- from UK daughters, ITB- from UK and foreign daughters, COM- foreign daughters only). HBN - bulls herd book number. Dtrs - number of daughters on which the proof is based. Herds - number of herds these daughters are in. % dtr top - Percentage of daughters, of the bull, milking in the herd with the most daughters. % top 2 - Percentage of daughters milking in the two herds with the most daughters. Lacts - the number of individual cow lactations used to calculate the proof. Rel % - The reliability of the Predicted Transmitting Abilities (PTA) in question. Milk (kg), Fat (kg), Prot (kg), Fat (%) and Prot (%) PTAs. SCC - Somatic Cell Count PTA (negative implies reduction in SCC). LS - Lifespan PTA. FI - Fertility Index. Indicates the financial benefit of improvement or reduction in fertility of the bull's daughters. Pers – Lactation Persistency PTA. Type - Indicates the type merit for the bull calculated from daughter linear and composite trait scores. £PIN - Production Index, Index combining Milk, Fat and Protein Yield PTAs. £PLI - Profitable Life Index. Like PIN but also includes longevity.

As shown above (*Table 1.1*) genetic evaluations usually contain the sire predicted transmitting ability (PTA) for production traits e.g. milk yield, fat, protein, fat % and

protein %. The selection indices widely used in the UK are based on the PTA and direct economic value of traits. The production index (PIN) combines PTAs for kg milk, fat and protein, the profitable lifetime index (PLI) combines PTAs for kg milk, fat, protein, lifespan, somatic cell count (SCC) and locomotion or feet and legs composite. Recently a female fertility index (FI) has been introduced in the UK, based on the calving interval and non return rate 56 days after insemination (NR56), PTAs being weighted according to their relative economic value (FI = X*Calving interval + Y*NR56 where X and Y are relative economic values in pounds sterling(£); Wall *et al.,* 2003a). To estimate calving interval and NR56 PTAs six traits are analysed together; these are: calving interval, NR56, interval to first service, number of inseminations per conception, body condition score (BCS), and milk yield, the latter two because of their genetic correlation to fertility and their higher heritability (heritability ± standard error, 0.237 ± 0.008 n = 12866; 0.329 ± 0.003 n = 43029 respectively, Wall *et al.*, 2003b). The FI is measured in pounds sterling and a positive value indicates better fertility and a financial benefit. In the future endocrine measures e.g. interval to commencement of luteal activity postpartum (CLA) may be incorporated into the fertility index because they have a higher heritability and are less susceptible to bias than calving interval and non return rate (Royal *et al.*, 2000b, 2002; Section 1.6).

1.2.2 The Danish dairy industry

In 2004 Denmark had approximately 569,000 dairy cows (71% Danish Holstein, 11% Danish Jersey, 8% Red Dane and 10% other) from 6600 herds with average size of 86 cows and milk yield of 7889 kg/cow/year (Danish Cattle Federation, Aarhus N, Denmark, 2005). Breeding schemes are similar in Denmark to those in the UK.

1.2.2.1 Selection indices

In Denmark genetic proofs, calculated by the Danish Cattle Federation (Aarhus N, Denmark), display breeding values for individual traits and values for the common selection indices used (*Table 1.2*). The Danish dairy industry has an overall index for

total merit which combines information on production, meat quality, daughter fertility, calving ability, udder health, health traits, body, feet and legs, mammary, milking speed, temperament and longevity (Dansire International, 2005). The three main indices relating to reproduction are the female fertility index, calving index and birth index. The calving index describes the mother's ability to have easy calvings and give birth to live born calves (maternal trait) and the birth index describes the sire's ability to breed easy born and live born calves (direct trait). The fertility index links information on several fertility measures (days from first to last insemination in heifers and cows, days from calving to first insemination in cows, non return rate in heifers and cows, heat strength in heifers and cows and fertility treatments in cows) which are combined and weighted according to economic value. The breeding values for female fertility are standardized to an average of 100 with a standard deviation of 5. Breeding values are estimated four times a year and a rolling base is used which includes all AI bulls born 5 and 6 years before publication (Danish cattle Federation, Aarhus N, Denmark, 2003).

Table 1.2 Example of the genetic evaluations produced for bulls in Denmark (Danish Cattle Federation, Aarhus N, Denmark).

HB no.	Name	Sire	MGS	Milk, index	Milk, kg
XXXX	XXXX	Luxemburg	R Leadman	119	+1117
XXXX	XXXX	Lukas	M Aerostar	94	-110
XXXX	XXXX	E Mattie G	N Luke	121	+1203
XXXX	XXXX	Manat	Lord Lily	117	+989

Fat, index	Fat, kg	Protein, index	Protein, kg	Yield	Calving ability	Daughter fertility	Birth
124	+54	135	+55	137	114	92	118
121	+48	104	+11	110	115	114	96
111	+28	135	+54	133	107	94	97
120	+48	121	+35	122	116	92	100

Longevity	Udder health	Other health traits	Body	Feet and legs	Mammary	Total Merit
113	110	119	101	122	96	140
121	128	118	99	113	115	132
110	100	103	110	93	111	132
	115		105	112	119	132

Additional Important Information

MGS – Maternal grandsire of the bull. Milk, fat, protein and yield index – production indices in standardized units compared to a certain base population/year. Milk kg, Fat kg & Protein kg – The breeding value for the total milk, fat and protein production based on the 305 day yield and upto the first 3 lactations. Calving ability, daughter fertility, birth, longevity, udder health, other health traits, body, feet & legs and mammary – these are relative, standardized indices compared to a certain base population. Total merit – the sub indices above are combined and weighted accordingly to give a total merit index.

1.2.3 Future technologies

New technologies are being developed that will further change the way breeding companies function. Such developments include: cloning, *in vitro* embryo production (IVEP), semen sexing which is carried out by some companies e.g. Cogent Breeding Ltd. (Cheshire, UK), marker assisted selection (MAS), gene assisted selection (GAS) and genetic modification of cattle. Implementation of some of these techniques could further increase the genetic progress possible.

1.3 THE JUVENILE COW

1.3.1 Prepubertal growth and development

Growth and development post weaning in the male and female is controlled largely by nutrition, health, genetics and management. Sufficient growth and development are prerequisites for the development of sexual function.

1.3.1.1 The female calf

Desjardi & Hafs (1969) studied reproductive organ growth, development and maturity in heifers fed a standard diet. In their study monthly slaughter (birth until ovarian cycles began) showed that the ovaries grew in size until 4 months of age, the growth stabilized until 8 months and then increased until 10 months of age at which point serial slaughter ended. The cervix and vagina grew slowly until 4 months of age and then rapidly until

the first ovulation, whereas the uterus showed a steady increase in size from birth until first ovulation (Desjardi & Hafs, 1969). The uterus continues to increase in size after first ovulation until approximately 3 years of age.

Honaramooz *et al.* (2004) studied Hereford heifers (n = 5) from birth until first ovulation (63.7 ± 1.1 weeks of age) and performed ultrasonographic examinations every 2 weeks. After birth the number of antral follicles increased rapidly until 4 months of age after which the increase stabilizes (Honaramooz *et al.*, 2004). The numbers of follicles ≥3mm diameter increased from 8 months of age until first ovulation (*Figure 1.3;* Honaramooz *et al.*, 2004) whereas follicle size increased steadily from birth until first ovulation (*Figure 1.4*; Honaramooz *et al.*, 2004). Follicular waves in prepubertal heifers occur in a similar way to mature cows in that they are initiated by a rise in follicle stimulating hormone (FSH) and the growing follicles produce oestradiol. The size and number of follicles, in addition to the oestradiol produced by the follicles, increases in the months preceding first ovulation (Honaramooz *et al.*, 2004; *Figure 1.3 & 1.4*).

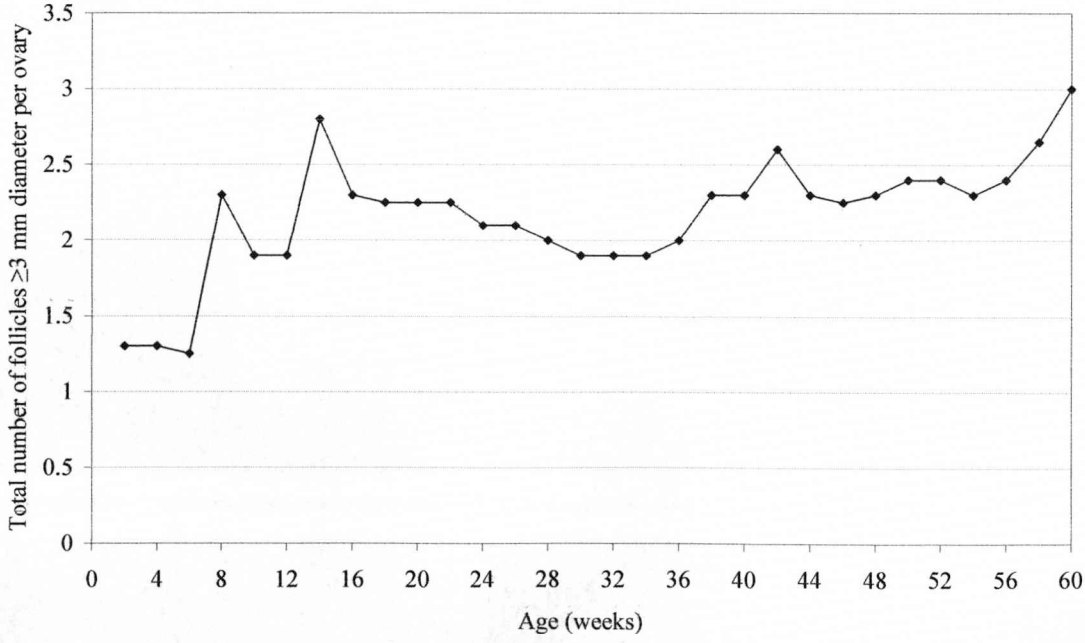

Figure 1.3 Total number of follicle ≥ 3mm in diameter in heifers from 2-60 weeks of age (redrawn from Honaramooz *et al.*, 2004).

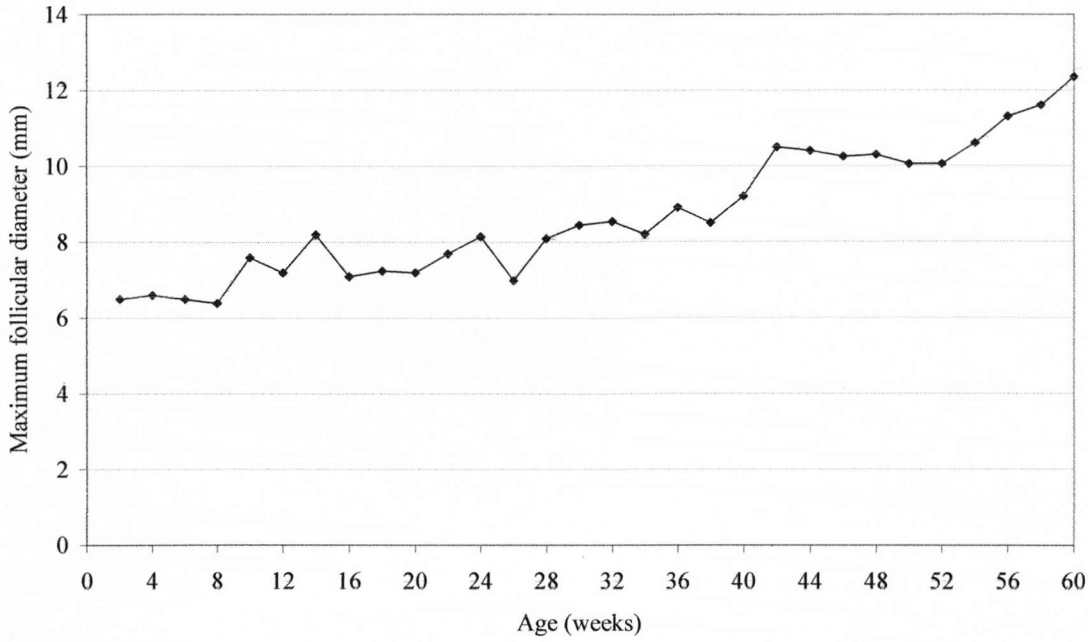

Figure 1.4 Maximum diameter of the largest follicles in heifers from 2-60 weeks of age (redrawn from Honaramooz *et al.*, 2004).

Luteinizing hormone (LH) and FSH plasma concentrations increase rapidly from birth and they remain high until 3 months of age, concentrations then fall and rise gradually until puberty (Rawlings *et al.*, 2003). The increasing concentrations of LH and FSH stimulate and maintain the growth and increase in numbers of antral follicles before puberty (Rawlings *et al.*, 2003).

1.3.1.2 The male calf

From birth until 10-12 weeks of age, the bull calf is in a period of infancy, there is virtually no gonadotrophin releasing hormone (GnRH) release from the hypothalamus (Amann, 1983). As a result LH pulses are infrequent, approximately one per 25 hour period, and concentrations are low. During infancy steroidogenesis in the Leydig cells is minimal (Amann *et al.*, 1986).

Curtis and Amann (1981) studied growth rate, development and establishment of spermatogenesis in Holstein bulls by castrating calves (total n = 52) from 12-32 weeks of age at 4 week intervals. They found that from birth to 32 weeks of age body weight increases rapidly and is highly correlated to age. At 12 weeks of age, the beginning of the prepubertal period, bull calves weighed approximately 79 ± 3 kg, scrotal circumference was 13.1 ± 0.4 cm and left testis weight was 9 ± 1 g. From 12-32 weeks of age, during the period of castrations, the scrotal circumference and left testis weight increased. At 32 weeks of age scrotal circumference was 26.8 ± 0.7 cm and left testis weight was 117 ± 10 g (Curtis & Amann, 1981). One of the criteria for puberty is scrotal circumference of 27.9 ± 0.2 cm and as puberty in Holstein bulls occurs at approximately 39-41 weeks of age, little growth must occur between 32 weeks of age and puberty (Amann, 1983).

Controlling the growth of the testes and establishment of spermatogenesis, in addition to nutrition, are changes in gonadotrophin secretion. The secretion of GnRH from the hypothalamus increases during the prepubertal stage (10-12 weeks of age until puberty) and the amount of GnRH receptors in the anterior pituitary increases (Rodriguez & Wise, 1991). LH pulse frequency and plasma LH concentration increases between 10-20 weeks of age then falls slightly until approximately 24 weeks of age then rises again to adult concentrations at puberty (Rawlings *et al.*, 1978; Amann, 1983). The early increase in LH causes the testes to grow and Leydig cells to begin to differentiate (Evans *et al.,* 1995). LH binds to receptors on Leydig cells and stimulates testosterone production. Because LH is released in a pulsatile manner the resulting testosterone production is also pulsatile (Amann, 1983). Testosterone concentrations rise from birth through to adult concentrations at around puberty; the increase is gradual until 15-25 weeks of age and more rapid afterwards (Rawlings *et al.*, 1978; Amann, 1983; Amann *et al.*, 1986). Testosterone is important in the normal functioning of spermatogenesis in the seminiferous tubules (Amann, 1983). FSH release increases from birth gradually until puberty and it binds to receptors on the Sertoli cells and is involved in spermatogenesis (Amann, 1983). It can be seen that LH, FSH and testosterone are very important in the initiation of spermatogenesis in the prepubertal bull.

1.3.2 Puberty

Puberty in the cow is characterised by the first behavioural oestrus accompanied by ovulation. The first ovulation in heifers is not usually accompanied by oestrus, the corpus luteum produced is small and the oestrous cycle is short (Rawlings *et al.,* 2003). The second ovulation does usually occur after oestrus and the cycle is of normal length (Rawlings *et al.,* 2003).

In the bull calf, puberty is considered to have been reached when scrotal circumference is 27.9 ± 0.2 cm and the ejaculate has 5.0 x 10^7 sperm/ml with progressive linear motility of 10% (Lunstra *et al.*, 1978). The age at which puberty occurs in males and females is determined by several factors including breed, season, nutrition and weight.

1.3.2.1 Initiation of puberty in the female

In general, puberty in the female occurs between 9 - 13 months; puberty tends to occur sooner in dairy breeds e.g. Holstein, Jersey and later in beef breeds e.g. Limousin, Charolais due to genetic differences and different selection pressures historically e.g. selecting for earlier puberty (Martin *et al.*, 1992). The weight of the heifer appears critical for the onset of puberty. The age at which puberty occurs can be lowered by feeding a high plane of nutrition which increases daily live weight gain and thus the critical weight is reached sooner (Chelikani *et al.,* 2003). However, early puberty resulting from a higher growth rate has been associated with lower milk yield due to insufficient mammary growth (Stelwagen & Grieve, 1990). In female Holsteins the weight at which puberty is initiated is between 250 and 350 kg (Martin *et al.*, 1992).

Gonadotrophins, in addition to controlling follicle growth in the pre- and postpubertal heifer, control the initiation of puberty (Day *et al.*, 1984). The LH pulse frequency just prior to puberty is crucial in increasing follicle growth which in turn increases oestradiol production and leads to the pre-ovulatory LH surge (Rawlings *et al.*, 2003). The increase in LH pulse frequency is partially due to the reduced negative feedback effect

of oestradiol on LH pulses in the 6 weeks before ovulation (Day *et al.*, 1984; 1987). In the prepubertal heifer low concentrations of oestradiol have a negative effect on GnRH release preventing LH pulse frequency increasing. The mechanism by which the negative effect of oestradiol on LH works is not exactly known. Endogenous opioids are thought to have an inhibitory effect on LH secretion in the heifer calf but this effect is not significant in the prepubertal period (Evans *et al.*, 1992).

In the weeks before puberty the number and size of follicles increase and so does oestradiol production (Rawlings *et al.,* 2003; Honaramooz *et al.,* 2004). The number of oestradiol receptors in the hypothalamus decreases in the 6 weeks before ovulation (Day *et al.,* 1987) which may reduce the sensitivity of the hypothalamus to negative feedback thus allowing LH pulse frequency to increase.

Another possible influence on LH pulse frequency before puberty are excitatory amino acids (Honaramooz *et al.,* 1999). Excitatory amino acids are excitatory neurotransmitters in the central nervous system (Honaramooz *et al.,* 1999). Honaramooz *et al.* (1998) showed that N-methyl-D, L-aspartic acid (NMA), an excitatory amino acid agonist, causes an instant release of LH and a smaller release of FSH in prepubertal heifers. Excitatory amino acids cause the release of GnRH which stimulates gonadotrophin release. Nitric oxide, a free radical produced from arginine by nitric oxide synthase, has also been shown to be involved in LH release in prepubertal rats (Honaramooz *et al.*, 1999). Nitric oxide synthase was found in the hypothalamus of rats in the region of GnRH neurones (Honaramooz *et al.*, 1999). Since arginine is the precursor of nitric oxide, nitric oxide may act as a metabolic signal linking the nutritional status of the animal to puberty onset. Prepubertal rats fed a diet lacking arginine showed delayed onset of puberty (Pau and Milner, 1982). Also in prepubertal ewes arginine was shown to stimulate LH release (Recabarren *et al.*, 1996).

The effect of nitric oxide was examined in an experiment in prepubertal (33 weeks) Hereford heifers subjected to four different treatments (Honaramooz, 1999). Group one (n = 5) were given N-G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase

inhibitor, group two (n = 5) were given N-methyl-D,L-aspartic acid (NMA), an excitatory amino acid agonist, group three (n = 5) were given L-NAME + NMA, group four (n = 5) were control animals which received saline, all compounds were given by intravenous injection once. Blood samples were collected every 15 minutes for ten hours and analysed for FSH and LH content. Results showed that L-NAME significantly ($P<0.05$) reduced the number of spontaneous LH pulses compared to animals receiving saline however no effect was observed on FSH secretion. Treatment with NMA resulted in significantly ($P<0.05$) higher LH release than the L-NAME + NMA group. The results indicate that nitric oxide is necessary in the process by which NMA causes LH release (Honaramooz *et al.*, 1999).

The mechanisms that allow LH pulse frequency to increase and initiate puberty are not fully known however it would appear to be an interaction between increased stimulation from excitatory amino acids and the reduced negative feedback effect of oestradiol on LH.

1.3.2.2 Initiation of puberty in the male

The age at which puberty occurs in male calves is generally between 39-53 weeks of age but varies according to breed, nutrition, growth and development. Rather than being a distinct event, puberty in the bull calf is the culmination of sufficient testes growth and sperm production. The rise in LH and FSH controls the development of the prespermatagonia until eventually spermatozoa are produced and spermatogenesis is occurring (Curtis and Amann, 1981). The mechanism by which LH and FSH increase after infancy is not fully understood. Amann *et al.* (1986) investigated how GnRH release and thus gonadotrophin release increases. This study examined neuroendocrine tissue and jugular blood samples from Holstein bull calves (total n = 25) slaughtered at 1, 6, 10, 14 and 18 weeks of age. The concentration of GnRH in the hypothalamus and median eminence did not alter from 1-18 weeks of age. However, GnRH receptor concentrations in the anterior pituitary more than tripled between 6-10 weeks of age and LH concentration increased also. Between 6-10 weeks of age oestradiol receptor

concentrations in the hypothalamus fell significantly whereas concentration of receptors in the anterior pituitary doubled. Oestradiol and testosterone have a negative effect on the hypothalamus and anterior pituitary to suppress GnRH, LH and FSH release (Amann, 1983). The alteration of receptor concentrations may indicate that the inhibitory effect of oestradiol in the hypothalamus on GnRH release is reduced. Thus the sensitivity of the anterior pituitary to GnRH increases greatly just prior to the prepubertal stage (Amann *et al.*, 1986).

The initiation of puberty in the male has also been associated with changes in the IGF system. At puberty there is evidence for an increase in insulin-like growth factor 1 (IGF-1), an increase in IGF-binding protein 3 (IGFBP-3) and a decrease in IGFBP-2 (Renaville *et al.*, 1996). The IGF system is recognised to be an important link between nutrition and reproduction which is most evident during periods of undernutrition where IGF-1 decreases, IGFBP-3 decreases and IGFBP-2 increases. This may have a detrimental effect on testosterone synthesis, semen quality and testis growth.

1.3.3 Factors affecting puberty

1.3.3.1 Nutrition and weight

With adequate nutrition puberty occurs between 7 - 12 months of age in male calves and 8-13 months in female calves (Martin *et al.*, 1992). With a high plane of nutrition puberty occurs earlier and likewise with restricted feeding puberty can be delayed.

1.3.3.1.1 Female

Work by Stelwagen & Grieve (1990) in which Holstein heifers (n = 41) were fed a total mixed ration at different levels to achieve low, medium and high average daily gain showed that weight is crucial for puberty to occur. The low, medium and high groups reached puberty at an average age of 365, 313 and 305 days, respectively, and at similar

body weight (average 284.9, 283.7 and 298.0 kg respectively) indicating that it is the weight of the heifer that determines puberty rather than the age.

Feeding a high plane of nutrition and thus advancing puberty may mean that the age at which heifers could be bred would be earlier thus reducing the costs of rearing before milk production. However, hastening puberty by feeding a high plane of nutrition has been associated with poor milk yield and altered mammary growth (Gardner *et al.*, 1977; Little & Kay, 1979; Stelwagen & Grieve, 1990).

1.3.3.1.2 Male

Renaville *et al.* (2000) found that feed restriction minimises growth and delays puberty in Belgian Blue calves possibly by altering IGF-1, IGFBP-2 and testosterone concentrations. Calves (285-295 kg and 290 days of age) were fed a normal fattening diet (n = 4), a restricted diet (n = 4) and a very restricted diet (n = 4) where growth would be minimal. Diets were fed for 114 days; then all animals were fed the normal diet for 100 days. IGF-1 concentrations were higher in the normal group than in the restricted and very restricted group. Concentrations increased in the normal group between 42-142 days (330-430 days old) but remained constant in the restricted and very restricted groups until feed restriction stopped after which concentrations rose slowly. In the normal group IGFBP-2 was significantly lower than in the restricted and very restricted diet groups. IGFBP-2 was highest in the very restricted group (Renaville *et al.*, 2000).

To ascertain the level of sexual maturation during the Renaville study, testosterone was measured at day 72 and 142 (1 month after feed restriction stopped) over an 18 hour period. The restricted and very restricted diet groups had no testosterone pulses during the 18 hour period whereas the normal group had approximately 2.35 pulses. The concentration of testosterone in the normal group was significantly higher than the restricted and very restricted groups (1.35 ± 0.35 ng/ml versus 0.71 ± 0.26 ng/ml and 0.17 ± 0.15 ng/ml; $P<0.05$). As testosterone plays a key role in testes growth and the

establishment of spermatogenesis, puberty onset in the restricted and very restricted groups will be altered. The normal group reached puberty sooner than the restricted and very restricted groups (day 71 of experiment in normal group, day 142-161 in restricted group and day 177 in the very restricted group). Once feed restriction ended testosterone concentrations increased, yet the very restricted group still had significantly lower concentrations than the two other groups suggesting that permanent damage had been done to the testes.

1.3.3.2 Season and photoperiod

There is evidence that season of birth and photoperiod affect the age at onset of puberty in calves. Possible factors involved include: autumn versus spring born, day length, diet, temperature and humidity. Various studies have been undertaken to determine whether the above affect puberty onset, however some results are conflicting. In some cases this may be due to differences in breed, place of study and management meaning that results are not comparable. The effect of season is likely to be due to photoperiod. Information regarding light and day length are relayed to the brain through the retina (reviewed by Dahl *et al.,* 2000). An inhibitory signal from the photoreceptors in the retina travels via the retina-hypothalamic tract to the pineal gland (Dahl *et al.,* 2000). At the pineal gland light inhibits melatonin production by inhibiting a key enzyme involved. During the dark the inhibition is removed and melatonin is produced (Dahl *et al.,* 2000).

1.3.3.2.1 Female

Honaramooz *et al.* (1999) examined the effect of season at birth on age at puberty in spring born (last week of March, n = 5) and autumn born (last week of October, n = 5) Hereford cross bred heifers in Canada. The spring and autumn born heifers reached puberty at a similar age and weight ($P>0.05$) however the spread of ages and weights in the autumn born heifers was much greater. Spring born heifers had higher LH pulse frequency and amplitude at 18 weeks of age than the autumn born heifers. In the spring born, LH pulse frequency increased until 32 weeks whereas in autumn born heifers LH

frequency peaked at 6 weeks and decreased at 12 and 18 weeks. FSH pulse frequency was higher at 12 weeks in spring than autumn born heifers. Although in this study the average age and weight at puberty was not significantly ($P>0.05$) different there were significant ($P<0.05$) differences in both LH and FSH profiles which would appear to be due to season of birth yet in this study this did not affect puberty onset (Honaramooz *et al.*, 1999).

Hansen *et al.* (1983) conducted an experiment to assess whether exposure to daily artificial light for extended periods would alter the age at puberty in Angus heifers in the USA. Sixteen heifers born between April–July were randomly assigned to either 18 hours of light per day (group L) or natural photoperiods (group N) from 22 weeks of age until puberty. Heifers in group L reached puberty significantly ($P<0.01$) earlier than heifers in group N, and both groups reached puberty at a similar weight. Ovarian growth rate was faster for group L than group N. No difference was seen in LH profiles between the two groups. The results would indicate that light exposure from 22 weeks can hasten puberty in calves born between April-July (Hansen *et al.*, 1983).

Melatonin (produced in dark periods) administration appears to affect puberty onset and this was highlighted in study by Tortonese and Inskeep (1992). Twenty four Hereford heifers (105 ± 5 days old; 134 ± 3.5 kg body weight) born between February and March were randomly assigned to either a control or melatonin group which had a melatonin ear implant for 5 weeks. The results showed that heifers in the melatonin group reached puberty sooner than the controls but at similar body weights (Tortonese & Inskeep, 1992). Therefore, from this study melatonin given early in the summer of winter born heifers speeds up the onset of puberty. As melatonin is produced during dark periods, the treatment induced short days which perhaps were perceived as the advancement of autumn/winter after a short summer period and this hastened puberty.

It would appear that season and photoperiod does affect onset of puberty. However the effect of season could be due to a combination of things such as change in day length, seasonal differences in diet, subtle changes in management and temperature. Long days

are known to increase growth rate in cows which could give similar results to feeding a high plane of nutrition and thus advancing puberty (Dahl *et al.*, 2000).

1.3.3.2.2 Male

Season of birth affects the onset of puberty in males and this has been shown in many studies (Aravindakshan *et al.*, 1999; Aravindakshan *et al.*, 2000). Hereford x Charolais bull calves born in September (n = 5; kept inside and fed a standard ration after weaning) and April (n = 5; kept at pasture until weaning at 24 weeks and then kept inside and fed the standard ration) were compared. Spring born calves had significantly ($P<0.05$) higher LH concentration than autumn born calves at 4, 8, 12 and 18 weeks of age after which concentrations were similar. LH pulse amplitude was significantly ($P<0.05$) higher in the spring than autumn born calves until 24 weeks of age after which they were similar. FSH concentrations, bodyweight, scrotal circumference and age at puberty were not significantly different between the two groups. However, there was a larger range in age and weight at puberty in the autumn born calves than the spring born (38-56 weeks & 307-362 kg at puberty in autumn born calves versus 44-48 weeks & 330-345 kg at puberty in spring born calves; Aravindakshan *et al.*, 2000) which is similar to results of Honaramooz *et al.* (1999) in heifers. This would be important from a management point of view as one would be more certain of when and at what weight spring born calves would reach puberty.

An earlier study by Aravindakshan *et al.* (1999) examined data retrospectively for differences in gonadotrophin secretion in early (36.6-44.2 weeks of age) and late (46.4-48.9 weeks of age) maturing spring born Hereford x Charolais bull calves. LH concentrations and pulse frequency were significantly ($P<0.05$) higher in the early maturing than the late group at week 12, 14 and 16 and weeks 10 and 20 respectively. Between 16-36 weeks of age early maturing calves were heavier than late maturing calves. Scrotal circumference was greater after 14 weeks in the early maturing bulls than the late maturing bulls. This paper shows that there is considerable variation in weight, gonadotrophins and testes development leading to different ages at puberty in

calves born in spring and raised in the same way. The cause of this variation is most likely genetic, due to the different sire and dam of the calves.

1.3.3.3 Breed

Breed has an effect on the age at which puberty is reached. The differences due to breed are similar in heifer and bull calves. Dairy breeds in general appear to reach puberty sooner than beef breeds (Martin *et al.*, 1992). This may be due to different selection pressures historically i.e. milk production versus growth rate.

The effect of breed, diet and season on puberty was examined by Grass *et al.* (1982). Hereford and Holstein June-born heifers (31 pairs) were compared at different planes of nutrition. Furthermore, autumn born heifers (total n = 62) from a Holstein dam and Angus, Hereford, Simmental or Chianina sire were fed two planes of nutrition and onset of puberty was monitored. In the first part of the experiment, Holsteins were younger and lighter than Herefords at puberty, both when fed at high and low planes of nutrition and both when winter or spring born. The results from the second experiment, where effect of breed of sire was being assessed, showed that heifers sired by Chianina were oldest at puberty followed by Simmental, then Angus and Hereford. Heifers on the high plane of nutrition reached puberty sooner than those on the low plane of nutrition.

The effect of breed of sire and dietary fat on puberty was examined by Lammoglia *et al.* (2000) in beef heifers. Heifers (total n = 246) bred from crossbred dams and either Hereford, Limousin or Piedmontese sires were fed low fat (1.9%) or high fat (4.4%) diets from 254 ± 4 days of age and the onset of puberty monitored. Heifers from Piedmontese sires were youngest at onset of puberty (343.7 ± 4.5 days) followed by Hereford sired (359.1 ± 4.7 days) followed by the oldest at puberty Limousin sired (379.8 ± 5.1 days). Furthermore, feeding a high fat diet in comparison to a low fat diet increased the onset of puberty most notably in Piedmontese sired heifers (349.2 ± 5.9 versus 338.1 ± 6.7 days respectively) than in Hereford or Limousin. These results would suggest that the effect of diet is not the same in all breeds. Breed of heifer and breed of

sire does affect the onset of puberty and although most work has been carried out in beef breeds it would seem logical to think that differences occur in the dairy breeds also.

Holstein bull calves reach puberty between 39-41 weeks of age (Amann, 1983). In Hereford bull calves puberty occurs between 39-52 weeks of age (Bagu *et al.,* 2004), in the Charolais between 33-53 weeks of age (Bagu *et al.,* 2004) and in the Belgian-Blue bull calf puberty occurs at approximately 51 weeks of age (Renaville *et al.,* 2000).

1.4 MATURE FEMALE REPRODUCTION

1.4.1 Oestrous cycles

1.4.1.1 Stages of the oestrous cycle

Cattle have consecutive oestrous cycles lasting approximately 21 days but this varies from 18-24 days (Macmillan & Burke, 1996; Royal *et al.*, 2000b). The oestrous cycle can be divided into the follicular phase and the luteal phase both of which are split into two stages (*Figure 1.5*). The follicular phase incorporates pro-oestrus followed by oestrus. The luteal phase incorporates metoestrus followed by dioestrus (Macmillan & Burke, 1996).

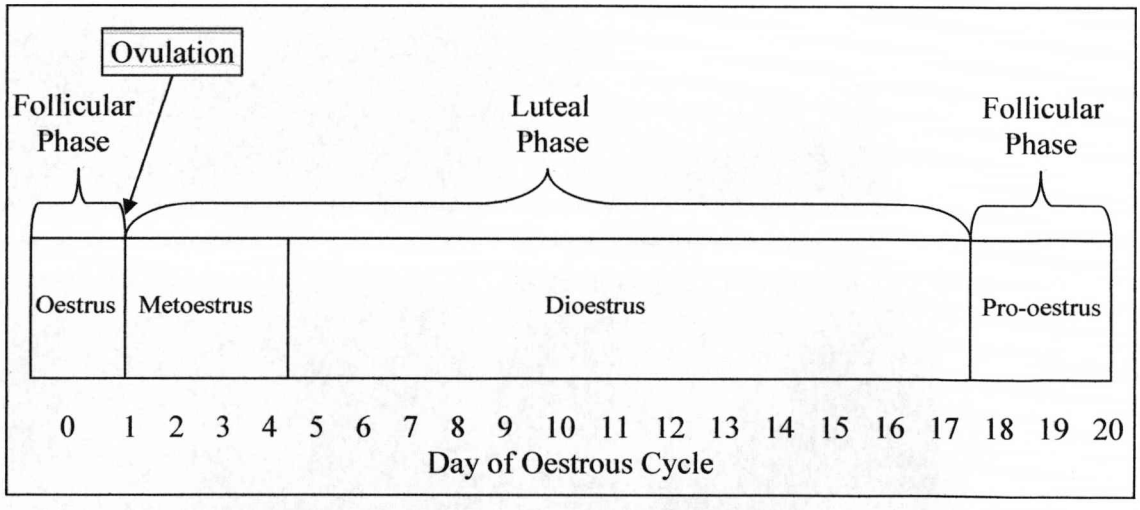

Figure 1.5 Stages of the oestrous cycle (adapted from Peters & Ball, 1995).

Oestrus occurs on day 0, lasts approximately 7 hours but can range from 6-30 hours in length and is the period when the cow is sexually receptive (Peters, 1985). Ovulation of the dominant follicle occurs on day 0/1 and is followed by metoestrus (day 1-4) the post-ovulatory stage during which the corpus luteum develops. During the dioestrus stage, day 5-18, the corpus luteum is secreting progesterone. Finally, there is the pro-oestrus stage, day 18-20, this is when the corpus luteum regresses and progesterone concentration falls (Peters, 1985).

1.4.1.2 Hormonal control of the oestrous cycle

Hormones secreted from the hypothalamus, anterior pituitary, ovaries and uterus control the stages of the oestrous cycle (Macmillan & Burke, 1996). *Figure 1.6* represents a schematic representation of the relative plasma concentrations of the main hormones involved.

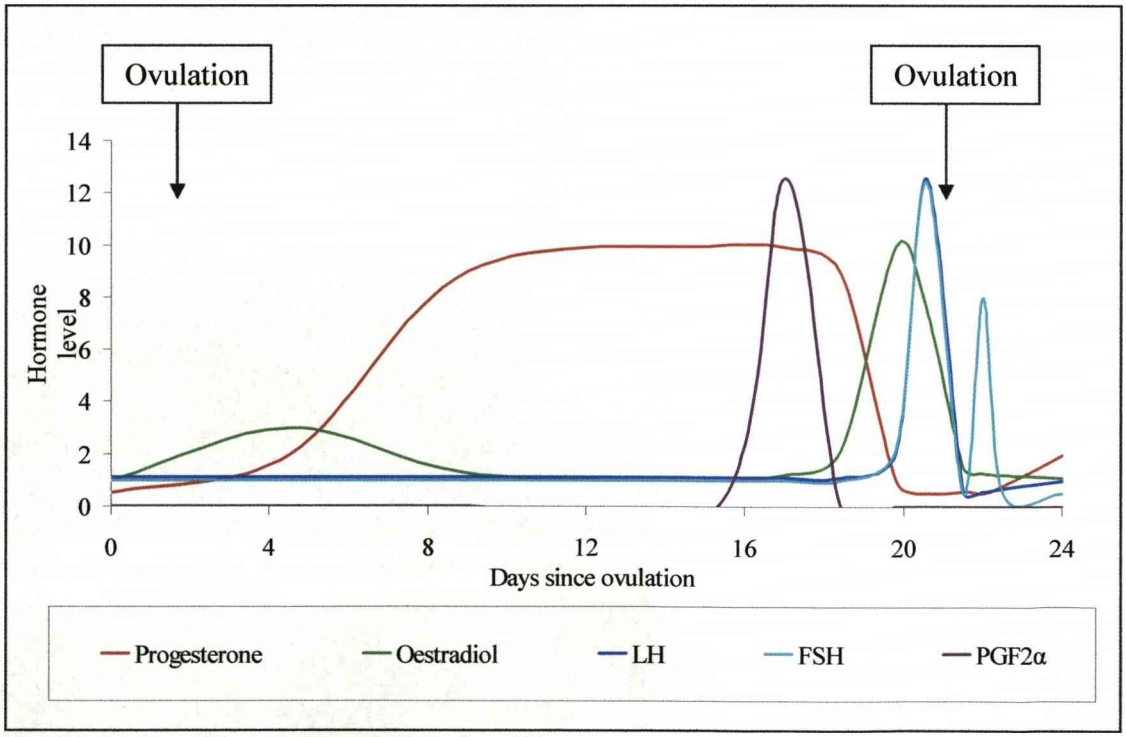

Figure 1.6 Plasma concentrations of hormones during the oestrous cycle (schematic; adapted from Peters, 1985).

The pre-ovulatory surge of LH from the anterior pituitary, as a result of increased GnRH from the hypothalamus, is thought to be the main luteotrophic signal (Milvae *et al.*, 1996; Hansel *et al.*, 1973). LH first initiates ovulation and then the development of the corpus luteum from the ruptured follicle. Many growth factor families e.g. vascular endothelial growth factor family, fibroblast growth factor family, insulin-like growth factor family and angiopoietin family are involved in the formation and regulation of the corpus luteum (reviewed by Schams & Berisha, 2004). The corpus luteum is present on the surface of the ovary between approximately day 1-4 to day 18-20 of the oestrous cycle. The granulosa cells in the ruptured follicle grow and develop into large luteal cells which secrete progesterone and oxytocin and are responsive to prostaglandin E (Hansel *et al.*, 1991). The theca interna layer develops into small luteal cells which also secrete progesterone but are responsive to LH (Hansel *et al.*, 1991). During the luteal phase, LH is secreted in a pulsatile manner at a low concentration which maintains the corpus luteum and stimulates progesterone synthesis in the small luteal cells. Growth hormone (GH), released from the anterior pituitary, stimulates progesterone and oxytocin synthesis by the large luteal cells (Schams & Berisha, 2004). The plasma LH concentration during the luteal phase remains low due to the negative feedback effect on the anterior pituitary and hypothalamus of both progesterone and oestradiol (Milvae *et al.*, 1996). The extremely vascularized nature of the corpus luteum allows most of the large and small luteal cells to be in contact with a capillary which aids the secretion of progesterone (Acosta & Miyamoto, 2004).

Progesterone concentration peaks at approximately day 8 and remains high until around day 17. At this time luteolysis occurs, involving the regression of the corpus luteum and the end of progesterone production. The main luteolytic hormone is prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Milvae *et al.*, 1996). Oxytocin receptor concentrations in the endometrium increase during late luteal phase which brings about an increase in the binding of luteal oxytocin. This is thought to exert a positive feedback effect on $PGF_{2\alpha}$ which in turn has a positive feedback effect on oxytocin (Milvae *et al.*, 1996). $PGF_{2\alpha}$ is secreted by the endometrium in a pulsatile manner and reaches the ovary by a local veno-arterial counter current transfer system (Hixon & Hansel, 1974).

When the corpus luteum regresses the progesterone concentration falls and the negative feedback on LH release is removed. In addition, oestradiol production from the dominant follicle increases and exerts a positive feedback effect to increase LH release from the anterior pituitary resulting in the pre-ovulatory LH surge (Milvae *et al.,* 1996). The increase in oestradiol also causes behavioural oestrus by an action in the hypothalamus (Allrich, 1994). Accompanying the LH surge is a FSH surge due to the increased release of GnRH by the hypothalamus. After ovulation there is a second rise in FSH which causes a group of small antral follicles to grow above 4mm in diameter and this forms the beginning of a follicular wave (Mihm *et al.*, 2002).

1.4.1.3 Folliculogenesis

At birth the cow has approximately 150,000 primordial follicles in the ovary, this number decreases over its lifetime due to wastage and as primordial follicles grow to a dominant follicle and ovulate or become atretic (Webb & Armstrong, 1998). The growth of primordial follicles occurs in a wave-like pattern with two or three successive waves occurring during each oestrous cycle (Sirois, J. & Fortune, J.E., 1988; Mihm *et al.*, 2002). Each follicular wave and subsequent recruitment of a group of primordial follicles to begin growth is initiated by a small rise in FSH (Fortune *et al.*, 2004). The FSH concentration falls over the next 2-3 days, as the amount of FSH falls the number of follicles from the original cohort still growing decreases until just one, the dominant follicle, is growing and the other follicles become atretic (Mihm *et al.,* 2002).

During this time the oestradiol production by the cohort of follicles alters. The smaller, slower growing follicles produce less oestradiol than the follicle which is destined to become the dominant follicle. Eventually when the dominant follicle is selected the remaining follicles become atretic and stop producing oestradiol (Mihm *et al.,* 2002). The dominant follicle continues to grow in response to LH; it continues to produce oestradiol which prevents a rise in FSH and therefore the recruitment of any other small follicles (Mihm *et al.*, 2002). However after 3-4 days, between day 5-8 of the oestrous cycle, the dominant follicle stops producing oestradiol and becomes atretic due to the

rise in progesterone from the developing corpus luteum and the negative feedback effect on LH pulse frequency (Mihm *et al.,* 2002).

Work by Gong *et al.* (1996) showed that by suppressing either FSH or LH release follicle growth was suspended at different stages. They concluded that FSH is needed during the 4-9 mm stage and LH is needed for growth beyond 9 mm (Gong *et al.,* 1996). After the first dominant follicle becomes atretic a second follicular wave is initiated by a rise in FSH, the dominant follicle will again become atretic. It is at the end of the third follicular wave that the dominant follicle will ovulate. This is due to the fall in progesterone at luteolysis which removes the negative feedback effect on LH and allows the pre-ovulatory LH surge (Milvae *et al.,* 1996).

1.4.1.4 The hypothalamus

The hypothalamus is located at the base of the brainstem behind the point at which the optic nerves enter the optic chiasma (See *Figure 1.7*; O'Connor, 2007). The hypothalamus contains different types of nerve cell bodies including the paraventricular nucleus, the supraoptic nucleus, the ventromedial nucleus and the arcuate nucleus which play important roles in reproduction. The hypothalamus is involved in many physiological systems including the control of body temperature, appetite and the pituitary gland (Mazzoccoli *et al.*, 2004). It is the hypothalamic control of the pituitary gland which is of most interest in reproduction.

The hypothalamus produces GnRH, in addition to other hormones, a polypeptide made up of ten amino acids that is central in the control of reproduction (Matsuo *et al.,* 1971). GnRH, released in a pulsatile manner from the hypothalamic neurosecretory cells, travels via the hypothalamo-hypophyseal portal vessels to the anterior pituitary where it binds to receptor cells to stimulate the synthesis and release of FSH and LH (reviewed by Herbison, 1997).

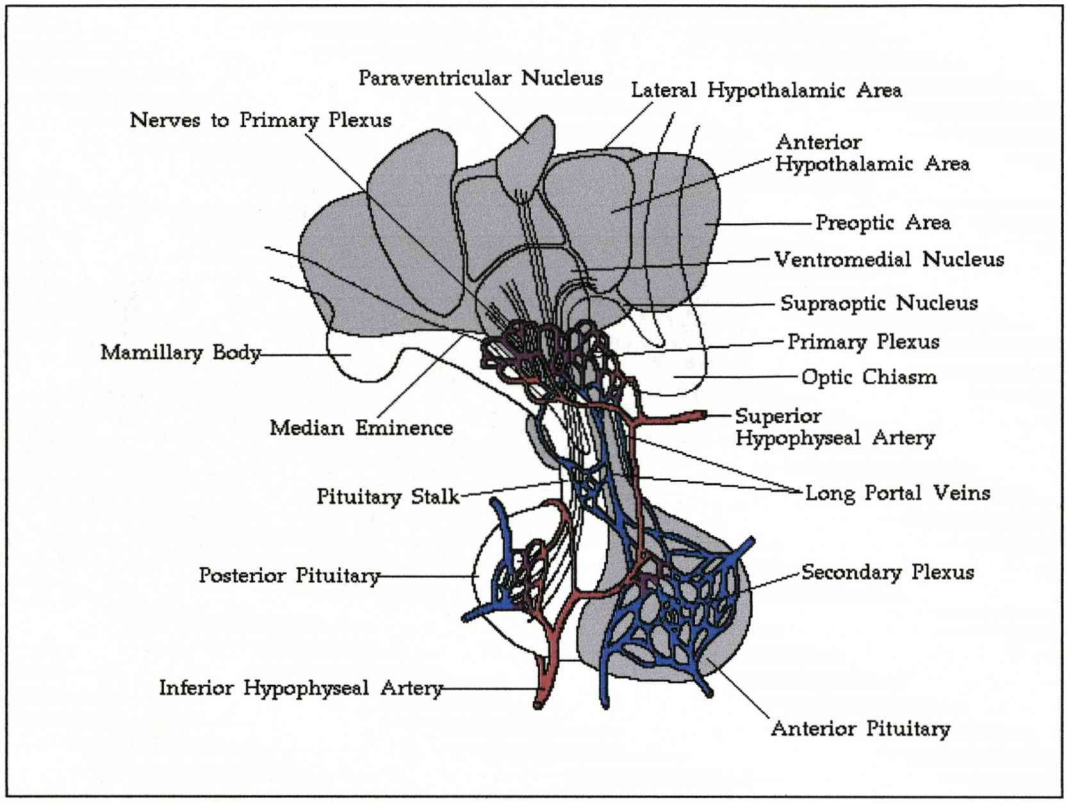

Figure 1.7 The hypothalamus and pituitary glands (O'Connor, 2007).

1.4.1.5 The pituitary gland

The pituitary gland is divided into two lobes, the anterior and the posterior pituitary (*Figure 1.7*). The posterior pituitary releases oxytocin and vasopressin (Leng & Brown, 1997). Oxytocin is involved in lactation, parturition and oestrous cycles. Vasopressin, also known as anti-diuretic hormone, acts in the kidneys to stimulate the re-absorption of water (Leng & Brown, 1997). From a reproductive point of the view the anterior pituitary is the more important of the two because it synthesizes major controlling hormones.

The anterior pituitary gland synthesizes and secretes LH and FSH in response to GnRH secreted from the hypothalamus (reviewed by Herbison, 1997). GnRH binds to high affinity receptors in the anterior pituitary which brings about the synthesis and release of LH and FSH. LH and FSH control folliculogenesis and ovulation during oestrous cycles

and are important for return to cyclicity postpartum and at puberty. The anterior pituitary also secretes GH, TSH, prolactin and adrenocorticotropic hormone (ACTH; Besser & Mortimer, 1974). Growth hormone is involved in growth and it is also involved in follicle growth by increasing IGF-1 production. TSH stimulates the thyroid gland to secrete hormones such as thyroxine and triiodothyronine which are important in growth and metabolism. Prolactin is involved in milk production and is released in response to suckling or stress (Bodnár *et al.*, 2004). ACTH acts on the adrenal cortex to cause the secretion of adrenal cortical hormones (Besser & Mortimer, 1974).

1.4.1.6 The insulin-like growth factor system

The IGF system is important in reproductive control particularly during oestrous cycles and during pregnancy. The IGF family consists of several related polypeptides: IGF-1, IGF-II, IGF binding proteins 1-6 (IGFBP1-6), several low affinity IGFBP-related proteins (IGFBP-rPs) and IGF type 1 receptor (IGF-1R) have been identified to date (Webb & Armstrong, 1998). Gene expression for all of the IGF family has been found in the ovary (Webb & Armstrong, 1998). IGF-II is produced in the thecal cells in the antral follicle. IGF-1 receptors (IGF-1R) and insulin receptors are found in the follicle, both IGF-1 & II act via the IGF-1R. It is not yet known whether IGF-1 is produced in the follicle or simply acts via the circulatory IGF-1 binding to IGF-1R (Webb & Armstrong, 1998). IGF-1 and insulin stimulate steroid synthesis by the follicle and corpus luteum and cause granulosa cell proliferation (Mihm *et al.*, 2002). IGFBP's can increase the activity of IGF-1 & II by transporting them to a specific tissue where the IGF-1GFBP complex acts as a store or they can reduce the availability of the IGF by binding to it and thus preventing binding to IGF-1R (Webb *et al.*, 1999).

The concentrations of IGF-1, II, IGF-1R and IGFBP's vary during follicular waves. During the 2-3 days after the FSH rise, that initiated a follicular wave, the amount of IGFBP4 and 5 is higher in the intrafollicular fluid of the follicles that are not growing as quickly as the follicle that will become the dominant follicle (Mihm *et al.*, 2002). Three days after the FSH rise the dominant follicle is selected. It has low intrafollicular

concentrations of IGFBP's, more IGF-II is produced and more IGF-1 is available to bind to IGF-1R resulting in higher oestradiol production. The concentrations of IGFBP4 and 5 are higher in the atretic follicles and rise in the dominant follicle if it fails to ovulate and becomes atretic (Mihm *et al.*, 2002).

The IGF system links reproductive function with the nutritional status of the animal. The amounts of IGF-1 and II synthesised in the liver are determined by the concentrations of glucose, insulin and GH. It is known that during the period of negative energy balance (See Chapter 1.4.3.1 for details), approximately 6 weeks postpartum, GH synthesis by the anterior pituitary gland increases. Concentrations of circulating insulin decreases and the concentration of liver GH receptors decrease. This affects the liver and causes the amount of IGF-1 produced to decrease (Webb *et al.*, 1999). Nutrition can affect the concentration of circulating IGF-1 of liver origin but it is also thought to affect the concentration of IGF-1 and II in the ovary.

1.4.2 Pregnancy

1.4.2.1 Maternal recognition of pregnancy

The blastocyst must signal its presence to its mother in order to prevent luteolysis. In the cow maternal recognition of pregnancy occurs at approximately day 17 (*Figure 1.8*). The elongated free blastocyst secretes a protein, between days 16-24, called interferon tau (IFNτ) which is a member of the cytokine family (Demmers *et al.*, 2001). IFNτ binds to receptors on the surface of the epithelial cells of the endometrium (Flint *et al.*, 1992; Demmers *et al.*, 2001). IFNτ reduces the expression of oxytocin receptors in the uterus and stops luteal oxytocin binding. This in turn prevents arachidonic acid being converted into $PGF_{2\alpha}$ by cyclooxygenase (Flint *et al.*, 1992). It is a surge of $PGF_{2\alpha}$ which causes the corpus luteum to regress after day 17.

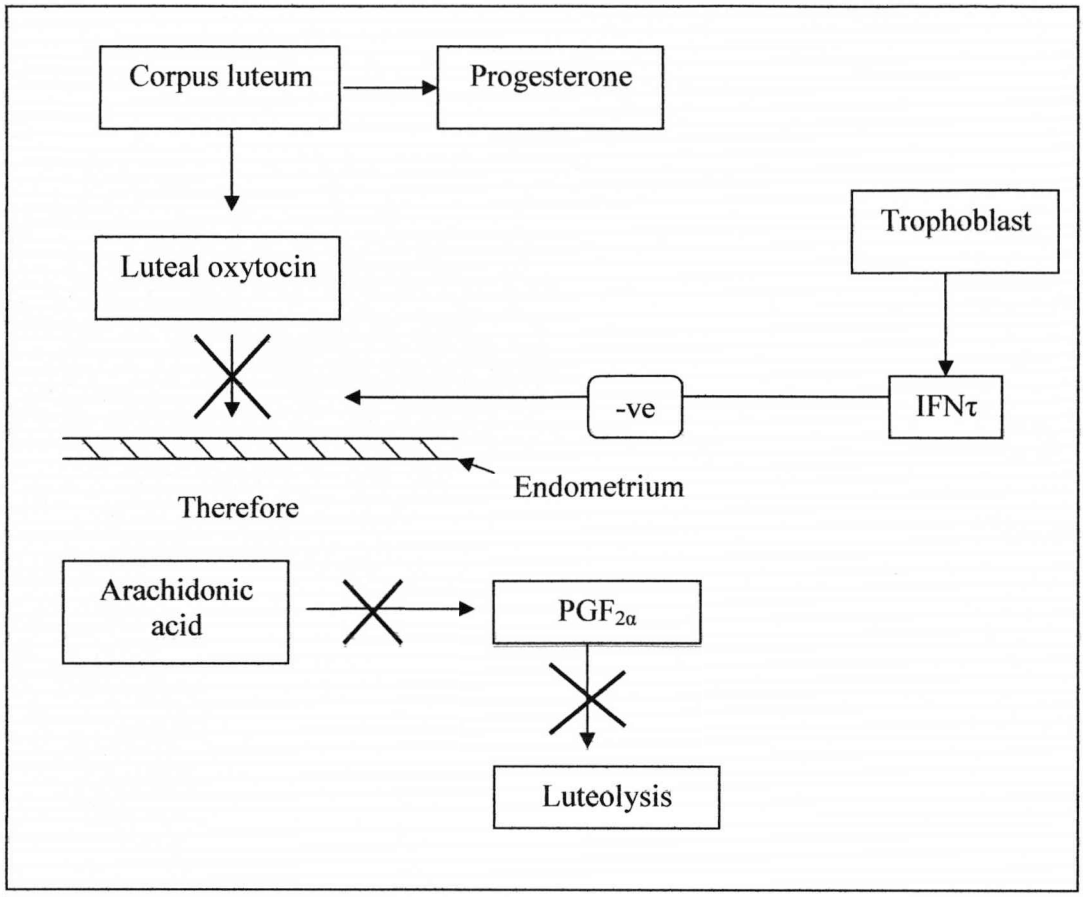

Figure 1.8 Sequence of events preventing luteolysis.

A review by Mann *et al.* (1999) estimated that failed maternal recognition of pregnancy and thus unsuccessful pregnancy occurred in 25-30% of inseminations. Early embryo mortality could occur because in these animals there may not be enough IFNτ produced to inhibit expression of the oxytocin receptors or IFNτ could be produced at the wrong time (Mann & Lamming, 1999). Progesterone is important in early embryo development and progesterone concentrations early in pregnancy determine the outcome. In 1971, Henricks *et al.* reported a lower concentration of progesterone on day 6 after mating in cows in which pregnancy failed in comparison to cows in which pregnancy was successful. Since then various studies have shown that lower concentrations of progesterone between day 12-17 post insemination and a delay in the post-ovulatory rise in progesterone are associated with subsequent pregnancy loss (Inskeep, 2004; Mann & Lamming, 1999). In 1998, Darwash and Lamming reported

that in cows with successful pregnancies milk progesterone was >3 ng/ml on day 4 after insemination compared to day 5 in cows with unsuccessful pregnancies.

1.4.2.2 Hormonal control of pregnancy

The corpus luteum secretes progesterone, which maintains pregnancy. In the cow the corpus luteum is the main source of progesterone for the first 200 days of pregnancy. After day 200, the corpus luteum can be removed and the pregnancy will remain this is thought to be due to the adrenal glands secreting progesterone and the placenta is also known to secrete progesterone (Kindahl *et al.*, 2004). Nevertheless, the corpus luteum is the most important source of progesterone and luteolysis occurs just days prior to parturition (Kindahl *et al.*, 2004).

Oestradiol, oestrone and oestriol are synthesised and secreted by the fetal membranes during pregnancy (Kindahl *et al.,* 2004). Oestrogens cause the myometrium to grow and the increase in muscle aids parturition. Oestrogens increase synthesis of actomyosin which is involved in creating contractility in the uterus. Oestrogen in conjunction with relaxin causes the cervix to soften, the birth canal to open and stimulates the release of $PGF_{2\alpha}$ from the endometrium at around the time of parturition (Kindahl *et al.,* 2004).

1.4.2.3 Dystocia

Dystocia is the term used to describe a difficult or prolonged parturition and is an important cause of fertility problems postpartum. Berger (1994) studied calving ease in the United States dairy industry and proposed a now commonly used scoring system (*Table 1.3*). The two main components of dystocia are the maternal effect i.e. the mothers ability to give birth and the direct effect i.e. the calf's ability to be born (Dekkers, 1994). Many factors influence the likelihood of dystocia occurring including genetics, parity of the dam, sex of the calf, weight of calf, season of birth. Through management many of the risk factors for dystocia can be controlled. Use of certain A.I.

bulls, breeds or sexed semen on heifers can reduce the likelihood of encountering calving difficulties.

Table 1.3 The calving ease scoring system (Berger, 1994).

Calving Score	Birth Difficulty
1	No problem
2	Slight problem
3	Needed assistance
4	Considerable force
5	Extreme difficulty

Dystocia represents an economic problem for the dairy farmer due to costs at calving e.g. drugs, veterinary surgeon and loss of calf or dam and afterwards as cows will often experience fertility problems. The most common fertility problem experienced, as a result of dystocia in part, is delayed resumption of ovarian activity (Dematawewa & Berger, 1997). Dematawewa & Berger (1997) analysed the effect of dystocia on 122,715 lactation records from 71,618 US Holsteins. They found that dystocia (scores 2-5) had a significant detrimental economic effect on 305d milk, fat and protein yields, days open, number of services and cow losses. The effect of dystocia increased as the score increased and was higher in heifers than in cows (Dematawewa & Berger, 1997).

1.4.3 Postpartum period: the return to cyclicity and negative energy balance

Following parturition the dry matter intake of a cow must increase 4-6 times to be able to meet the extra energy demanded for milk production (Roche *et al.,* 2000). However, for most cows, this is not possible immediately and therefore the extra energy demand for milk production can not be met by the diet alone resulting in body reserves being mobilized for milk production. This status is termed negative energy balance (NEB) and can last for several weeks following parturition. There is a great deal of variation in the length and severity of NEB and this is reported to be related to differences in genetic merit for milk yield, parity, milk yield, body condition score (BCS) at parturition and

dry matter intake (Mao *et al.*, 2004). During this period of NEB, changes are seen in the concentrations of free fatty acids (FFA), glucose, GH, insulin, IGF-1 and other regulatory hormones (Hart, 1983; Spicer *et al.*, 1990; Butler, 2000; Roche, 2000). The duration and severity of NEB postpartum is unfavourably genetically and phenotypically correlated with the interval to first ovulation (Butler, 2000; de Vries & Veerkamp, 2000; Dechow *et al.*, 2002; Royal *et al.*, 2002).

Approximately 5-7 days after parturition a follicular wave is initiated by a rise in FSH. This follicular wave either results in ovulation of the dominant follicle (16-20 days postpartum), the follicle does not ovulate and a new follicular wave begins or the dominant follicle does not ovulate and becomes cystic. The latter two can lead to the first ovulation postpartum after 40 or 50 days (Butler, 2000). Negative energy balance reduces LH pulse frequency which limits follicle growth and oestradiol production (Butler, 2000). Synthesis of GH by the anterior pituitary gland increases (Diskin *et al.*, 2003) causing an increase in lipolysis, which results in a raised amount of circulating FFA (Hart, 1983), of which some, in turn, is transported to the liver where it can accumulate and lead to liver ketosis (Bobe *et al.*, 2004). Concentrations of circulating glucose and insulin decrease. Furthermore, the concentration of liver GH receptors decrease which causes the amount of IGF-1 produced by the liver to decrease (Webb *et al.*, 1999; Butler *et al.*, 2003). The decreased amount of IGF-1 decreases the rate of follicle growth. This may lead to a smaller dominant follicle which produces less oestradiol and is therefore less able to exert a positive feedback affect on LH and cause the pre-ovulatory LH surge and thus ovulation.

1.5 THE DECLINE OF FEMALE FERTILITY

During recent years dairy cows have been selected for high milk yields and American Holstein genes have been introduced into the UK dairy herd (Miglior *et al.*, 2005). It was estimated that the proportion of American Holstein genes in the UK herd in 2000 was 80% compared to 0% in 1975 (Royal *et al.*, 2000b). Milk yield in the Holstein has increased greatly over the last 30 years with an average yield of 8212 kg/year reported in

2005 (NMR, Greenway Business Park, Belinger Close, Chippenham). The average herd size has also increased from 40 in 1975 (Royal *et al.*, 2000b) to 92 in 2003 (Milk Development Council) which in some cases has lead to poorer management (Webb *et al.*, 1999). Unfortunately the increase in milk yield and increase in herd size has been accompanied by a decline in fertility (Royal *et al.*, 2000b).

There are several ways in which the decline in fertility is evident. Royal *et al.* (2000a) compared reproductive measures between 1975 – 1982 (total n = 1683) and 1995 – 1998 (total n = 714) and found that the occurrence of persistent corpus luteum after the first ovulation increased (7.3% to 18.2%), interval to first service rose (73.9 to 77.6 days) and pregnancy rate to first service fell (55.6% to 39.7%; Royal *et al.*, 2000a). More recently, a study by Mayne *et al.* (2002) in Northern Ireland (n = 2471 cows) reported the average conception rate to first service was 37.1 %. A similar proportion of cows suffering from atypical ovarian hormone patterns was seen in a study by Opsomer *et al.* (1998) in Belgium. In this study milk progesterone was sampled, twice a week for 3 months or until pregnancy confirmed, in 335 high yielding Holstein-Friesians for 448 lactations. Of the cows studied 46.5% were suffering from one or more atypical ovarian hormone pattern during the first three months postpartum. 20.5% showed prolonged anovulation postpartum (defined as milk progesterone <15 ng/ml for ≥50 days postpartum), 3% showed prolonged interluteal phase (defined as milk progesterone <15 ng/ml for ≥14 days), 20% showed delayed luteolysis (defined as milk progesterone ≥15 ng/ml for ≥20 days) and 3% of the cows showed a combination atypical cycles (Opsomer *et al.*, 1998). The decline of female fertility in the dairy cow is not unique to the UK, similar trends are present in other countries e.g. Belgium (Opsomer *et al.*, 1998), USA (Lucy, 2001), Ireland (Evans *et al.*, 2006a), Spain (López-Gatius, 2003) and the Netherlands (Ouweltjes *et al.*, 1996).

Low fertility postpartum in the UK was estimated to cost £3 per cow per day assuming 60% of cows are infertile for 30 days per year, this represents lost income from milk sales, feed costs, quota costs, culling and replacement of subfertile cows and extra semen costs for animals that need to be inseminated several times (Royal *et al.*, 2000a).

It is evident that subfertility represents a serious problem to farmers not only financially but also from a welfare point of view as subfertile cows are inevitably culled early and replaced.

1.6 GENETICS OF FEMALE FERTILITY

1.6.1 Traditional parameters of fertility

Traditional parameters of fertility can be broadly divided into interval traits and fertility scores. Interval traits are measured as the number of days from one event to another and fertility scores are often percentages. Interval traits include:- days open - the number of days the cow is not pregnant after calving; calving interval - the number of days between two consecutive calvings; interval to first service - the number of days until the first service postpartum. Fertility scores include: conception rate to first service, number of inseminations per conception and non return rate, this is the proportion of cows that do not return to oestrus, usually 56 days after insemination.

Any of the above parameters could, in theory, be incorporated into breeding goals, however, there are problems associated with these traditional parameters. Firstly, to be able to measure any of the above, one must wait until the cow has had one calf, therefore until a minimum of 2 years old and for some, such as calving interval, until the second calf is born. To make the most improvement through selection, animals must be assessed as early as possible in order to determine their value and the value of their sire in the breeding programme. Also a problem is that the above fertility parameters can only be measured in the female (Royal *et al.*, 2000a). Another problem associated with these parameters is that recording of fertility data in the UK is not compulsory and therefore records kept of inseminations, conception and calving interval are sometimes incomplete (Royal *et al.*, 2002). Also the parameters can be biased by management for example preferential treatment of cows with good genetic merit. A further problem is that the traditional parameters of fertility have low heritability, typically $h^2<0.05$ (Hoekstra *et al.*, 1994; Pryce *et al.*, 1997). Large environmental variation, due to for

example management and diet, masks the genetic variation and makes it difficult to identify genetically superior animals except by using large progeny groups.

1.6.2 Endocrine parameters of fertility

Endocrine parameters of fertility, at present, are based on the measurement of progesterone. The concentration of progesterone changes during the oestrous cycle and progesterone is important during the early stages of pregnancy (Section 1.5.2.2). Both milk and plasma progesterone can be monitored in the cow to build up a picture of its reproductive status. Milk progesterone concentrations are more commonly used because they can be easily and regularly collected with little stress caused to the cow. Milk samples are assayed for their progesterone content using a milk progesterone enzyme linked immuno-sorbent assay (ELISA; Royal *et al.*, 2000b) or a radioimmunoassay (RIA; Lamming & Darwash, 1998). Milk progesterone concentrations, can identify ovulation, the length of the luteal phase, whether pregnancy is maintained and whether there are cycle abnormalities such as prolonged inter-luteal phase, delayed luteolysis or prolonged anoestrus postpartum (*Figure 1.9*; Royal *et al.*, 2000a).

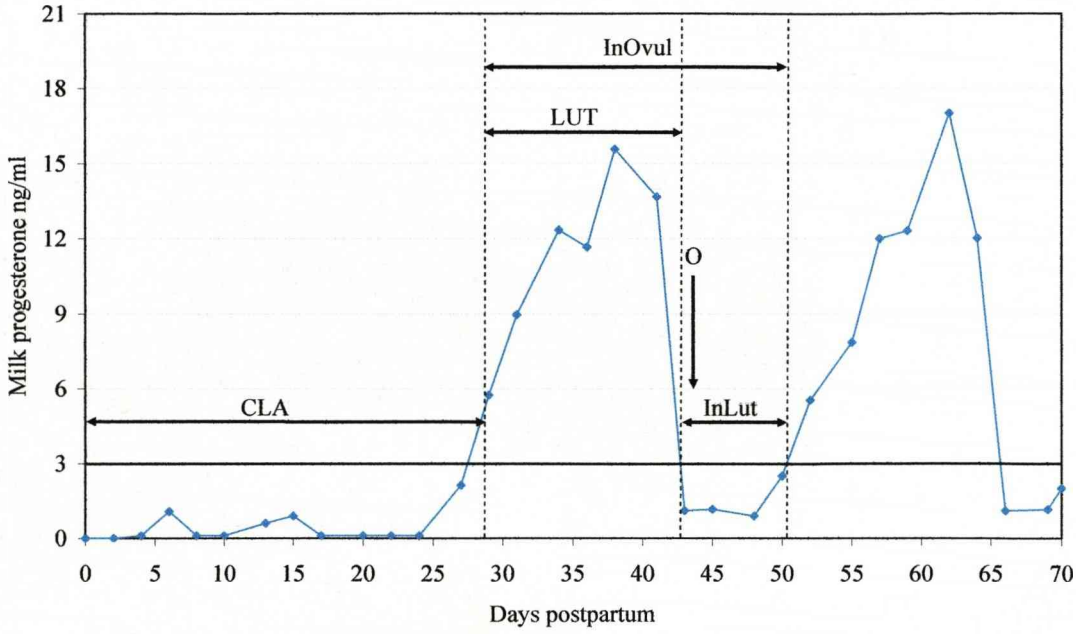

Figure 1.9 Milk progesterone profile showing reproductive parameters (CLA - Interval to commencement of luteal activity postpartum, Lut - Length of the luteal phase, InLut – Interval between consecutive luteal phases, InOvul – interval between consecutive ovulations and O – oestrus; adapted from Royal *et al.*, 2000b)

The endocrine parameters obtainable from milk progesterone measurement can be divided into interval traits and fertility scores.

Interval traits include:

Commencement of luteal activity (CLA) – The number of days postpartum until luteal activity (≥2 consecutive samples with milk progesterone ≥3ng/ml when milk sampling carried out 3 times per week).

Length of luteal phase (LUT) – the number of consecutive days following ovulation with progesterone ≥3ng/ml until progesterone falls to <3ng/ml.

Length of inter-luteal phase (InLut) – the number of days between the regression of one corpus luteum and the formation of a subsequent corpus luteum defined as the period from the first progesterone concentration <3ng/ml till the last consecutive progesterone concentration <3ng/ml.

Length of inter-ovulatory interval (InOvul) – The period between two ovulations defined as the number of days from the first progesterone ≥3ng/ml (followed by consecutive progesterone ≥3ng/ml representing a luteal phase) until the first milk progesterone ≥3ng/ml of the next luteal phase (Royal *et al.*, 2000b).

Fertility scores obtained from progesterone measurements include:

Delayed ovulation type I (DOVI) – Milk progesterone <3ng/ml for ≥45 days postpartum.

Prolonged inter-luteal interval, delayed ovulation type II (DOVII) – Milk progesterone <3 ng/ml for ≥12 days between two luteal phases.

Delayed luteolysis during the first cycle, persistent corpus luteum type I (PCLI) – Milk progesterone ≥3 ng/ml for ≥19 days during the first oestrous cycle postpartum.

Delayed luteolysis during subsequent cycles before insemination, persistent corpus luteum type II (PCLII) – Milk progesterone ≥3 ng/ml for ≥19 days during subsequent oestrous cycles postpartum (Lamming & Darwash, 1998).

Proportion of samples with luteal activity (PLA) – The proportion of samples with milk progesterone ≥3ng/ml when milk sampling is carried out 3 times per week during the first 60 days postpartum. PLA can be measured using a number of different milk sampling frequencies (three times per week, PLA_a; weekly, PLA_w; fortnightly, PLA_f; monthly, PLA_m; Petersson *et al.,* 2006a; 2006b).

Endocrine parameters of fertility overcome some of the problems associated with traditional parameters of fertility. Milk samples can be collected during milking therefore causing no additional stress to the cow. On farm milk progesterone kits are available which allow the farmer to test samples and have results quickly (NMR; Ridgeway Science Ltd., Alvington, Glocestershire, UK). Also the data are less biased by management or preferential treatment, however, any hormone treatments given during or before the sampling period may affect the results and may bias the data. This poses a problem with traditional measures of fertility also. Endocrine parameters also show greater heritability than traditional measures of fertility (*Table 1.4*).

Table 1.4 Heritability estimates for endocrine parameters of fertility

Endocrine Parameter of Fertility	**Heritability (Standard error)**
Interval to commencement of luteal activity	0.21 (n = 2349, Darwash *et al.*, 1997a) 0.16 (0.10, n = 329, Veerkamp *et al.* 2000) 0.16 (0.05, n = 1212, P<0.001; Royal *et al.*, 2002)
Length of luteal phase	0.17 (0.06, n = 1146, P<0.001; Royal *et al.*, 2002)
Persistent corpus luteum type I	0.13 (0.06, n = 1146, P<0.05; Royal *et al.*, 2002)

Furthermore, CLA has been found to be phenotypically and genetically correlated with other fertility parameters (*Table 1.5*).

Table 1.5 Genetic correlation (r_A) and phenotypic association ($b \pm$ standard error) between CLA and traditional fertility parameters.

Fertility parameter	**r_A**	**Reference**	***b* ± s.e**	**Reference**
Days to first service	0.53	a	0.24 ± 0.04	b
Calving interval	0.39	a	---	---
Days to conception	---	---	0.41 ± 0.09	b
Services per conception	0.048	a	0.0057 ± 0.0013	b

a Royal *et al.*, 2003

b Darwash *et al.*, 1997b

Due to the favourable correlations between CLA and for example calving interval selecting animals with a shorter CLA is likely to reduce calving interval (*Table 1.5*). Incorporating one of the endocrine parameters, such as CLA, into selection indices would possibly yield greater improvement in fertility over a shorter time in comparison to the traditional parameters of fertility because it can be measured sooner and with greater certainty.

Of the endocrine fertility parameters described CLA and PLA are perhaps the most useful and most widely studied (e.g. Darwash *et al.*, 1997; Royal, 1999, *et al.*, 2000b, 2002; Veerkamp *et al.*, 2000; Petersson *et al.*, 2006a; 2006b; 2007). However both CLA and PLA measures (PLA_a; PLA_w; PLA_f; PLA_m) need to be interpreted with caution. A short CLA is indicative of a earlier resumption of luteal activity and CLA is both phenotypically and genetically correlated with traditional fertility parameters such that cows with a short CLA have a shorter calving interval (*Table 1.5*). However, despite this it would not necessarily be advantageous to have the shortest CLA. Early postpartum ovulation (before 21 days) has been associated with poorer reproductive performance (longer interval from calving till conception, more services per cow and lower conception rate) in comparison to later ovulating cows (Smith & Wallace, 1998). It is likely that a short CLA, such as 27 days is most beneficial for resumption of normal ovarian function and fertility (Darwash *et al.*, 1997b).

The PLA fertility measures are complicated to interpret because the ideal PLA has not been determined, but suggested as being intermediate (Petersson *et al.*, 2007). PLA_a has been found to be negatively genetically correlated to delayed ovulation postpartum (genetic correlation ± standard error; -0.932 ± 0.055; Petersson *et al.*, 2007) whilst positively genetically correlated to persistent corpus luteum postpartum (0.662 ± 0.154; Petersson *et al.*, 2007). These results indicate that low PLA cows are likely to have delayed ovulation postpartum whereas high PLA cows may have extended luteal phases typical of persistent corpus luteum. A medium PLA is probably associated most often with normal resumption of cyclicity and therefore care would need to be taken if using PLA measures in selection indices. If we assume the average luteal phase is approximately 14.8 days (Royal *et al.*, 2000b), the average CLA is approximately 27 days (Darwash *et al.*, 1997b) and the average inter-ovulatory interval (i.e. cycle length) is 22 days (Macmillan & Burke, 1996; Royal *et al.*, 2000b) then an average PLA can be calculated (*Figure 1.10*)

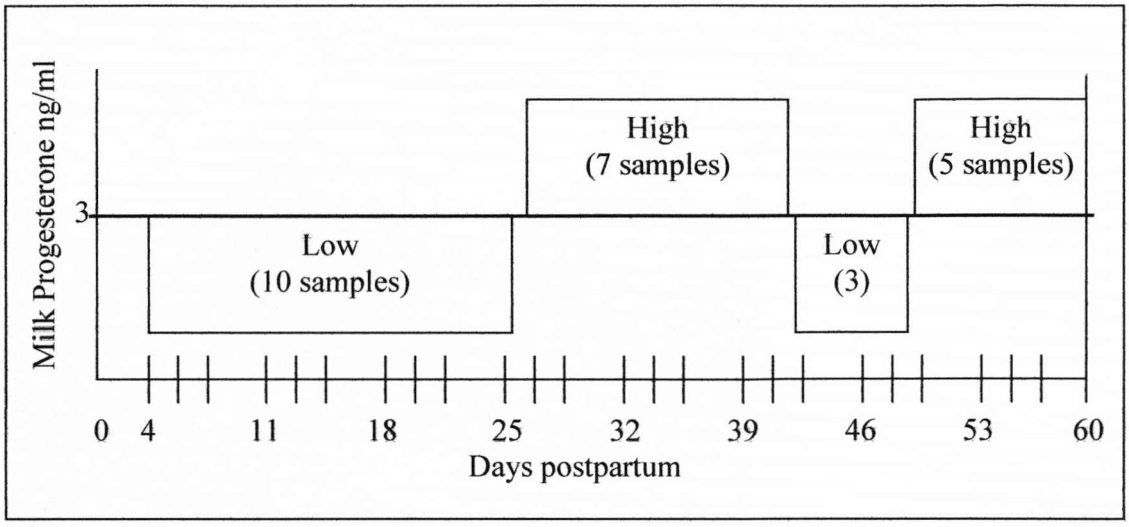

Figure 1.10 Illustration of the proportion of milk samples with luteal activity (≥3ng/ml) during 60 days postpartum with three times per week milk sampling and the first sample taken on day 4 postpartum. Example PLA = (7 + 5) / 25 = 0.48

With normal postpartum cyclicity the estimated PLA is 0.48 (*Figure 1.10*) therefore perhaps values outside the range 0.38 to 0.58 may be associated with atypical ovarian activity such as delayed ovulation or persistent corpus luteum respectively.

Endocrine parameters of fertility partly overcome the biological constraints associated with traditional parameters of fertility. Endocrine parameters can only be measured in the mature female but they can be measured by milk sampling from calving until necessary and one does not have to wait until the cow begins its second lactation.

The ideal fertility parameter to use in a breeding programme would be:

- Measurable in the male – The greatest genetic improvement is possible through the sire. Popular bulls produce thousands of offspring through artificial insemination whereas a heifer will only produce 4 or 5 offspring.
- Measurable at an early age – The rate of genetic improvement is determined by the generation interval i.e. the period of time from when an animal is born until it produces offspring. In the female and male the generation interval is a minimum of 20 months, however, in reality the generation interval in the male is

at least 4 years to allow the bull to be progeny tested and get a proof and even longer if the bull is to be used to breed bulls (Dekkers, 1992). Therefore the ideal trait would be measurable in a prepubertal animal to allow a decision to be made whether to use it in the breeding scheme, cull it or sell it without having to wait until it has offspring to make the decision.

- Moderately heritable – Another factor controlling genetic improvement of a trait is the heritability. The higher the heritability of a trait the more it is controlled by genetics rather than management and nutrition and therefore it is easier to identify genetically superior animals that are able to pass on there genetic merit for a trait to their offspring.
- Genetically correlated to female fertility – If the trait is to be measured in the prepubertal male then in order to improve female fertility it must be genetically correlated to adult female fertility (Falconer & Mackay, 1996b).

If a suitable juvenile predictor of female fertility was found in the male it could potentially have a huge impact on the rate of improvement of fertility. A fertility index has recently been introduced in the UK (Wall *et al.*, 2003a; Section 1.2) however the incorporation of a suitable juvenile predictor of female fertility in the male into the present index could improve rates of genetic improvement.

1.7 ESTIMATING GENETIC PARAMETERS

1.7.1 Family structure

When estimating genetic parameters it is important that the family structure is suitable. The family structure needed depends on the method of estimation that will be used and the predicted magnitude of the heritability to be estimated (discussed below). In order to get reliable estimates with small standard errors a large data set is needed.

1.7.2 Heritability

The heritability, of a trait in simple terms is the extent to which a trait is passed to offspring from the parents. It is denoted by h^2, first described by Wright (1921), and is the proportion of phenotypic variance attributable to additive genetic variance:

$$h^2 = \frac{V_A}{V_P}$$

Where V_A is the additive genetic variation and V_P is the phenotypic variance. An estimate of the heritability of a trait is most relevant to the population from which the data used for estimation was taken and becomes less reliable as an estimator for other populations.

The method used for estimating heritability is largely determined by the structure of the data available and in order to maximise precision and minimise bias. The heritability can be estimated by half or full sib correlation, regression of offspring on both parents, mid-parent or one parent (Falconer & Mackay, 1996a). Whichever method of analysis is going to be used one needs to decide beforehand how many families will be measured and the number of offspring per family. The number is usually limited by labour and cost of animals, rearing, housing etc. In order to make the correct decision the optimal design theory must be considered. Robertson (1959) described the optimal design theory which relates to the sampling variance of the intraclass correlation t and t is estimated in order to obtain the heritability (See below). Robertson discusses the optimal design for several methods of estimating the heritability but most relevant here is for half sib analysis which is most commonly used in cattle studies. The optimal design theory states that the sampling variance (variance of t) is minimal when nt=1 where n is the number of offspring per family and t is the intraclass correlation coefficient ($t = ¼ h^2$ in half sib analysis). For example, if we assume that the heritability to be estimated is likely to be between 0.04-0.4 then a family size (n) between 20-30 is optimal (Robertson, 1959).

The heritability is obtained by adjusting the regression coefficient (b) or the intraclass correlation coefficient (t) according to the relationship between the individuals used for the calculation (See *Table 1.6*).

Table 1.6 Association between the covariance between relatives, regression (b) or correlation (t) coefficient and heritability when estimated from different relatives (V_A = additive genetic variance; V_D = dominance variance; V_{Ec} = common environmental variance; Falconer & Mackay, 1996a).

Relatives	**Covariance**	**Regression (b) or correlation (t)**
Offspring and one parent	$\frac{1}{2} V_A$	$b = \frac{1}{2} h^2$
Offspring and mid-parent	$\frac{1}{2} V_A$	$b = h^2$
Half sibs	$\frac{1}{4} V_A$	$t = \frac{1}{4} h^2$
Full sibs	$\frac{1}{2} V_A + \frac{1}{4} V_D + V_{Ec}$	$t \geq \frac{1}{2} h^2$

The covariance or degree of phenotypic resemblance between relatives is an estimate of the additive genetic variance when adjusted for the class of relatives used e.g. ¼ for half sibs. The coefficient of relatedness (r) multiplied by the additive genetic variance gives the phenotypic covariance. The coefficient of relatedness (r) is the proportion of genes the relatives can have in common, for example an individual receives half of its genes from each parent. The covariance between full sibs is slightly more complicated due to the added variance due to dominance and the variance due to the common environment. This method of estimation can greatly overestimate the heritability and is most biased (Falconer & Mackay, 1996a).

When estimating genetic correlations and heritabilities with large data sets, it is easiest and most accurate to fit univariate models to the data, when analysing one trait or multivariate models, when analysing several traits at once. This is essentially regression with additional fixed effects e.g. age at calving, farm, sex and random effects fitted e.g. the random genetic effect of sire. See *Figure 1.11* for an example of a univariate model fitted to data to estimate genetic parameters.

$$Y_{ijklmno} = \mu_{kl} + YWR_{klm} + H_{kln} + AAC_{klo} + D_{ij} + S_j + e_{ijklmno}$$

Where, $Y_{ijklmno}$ is the ith observation for the jth individual of the kth trait at lth days in milk (DIM) at mth year and week of record in nth herd with oth age at calving; μ_{kl} is the mean for the kth trait at lth DIM; YWR_{klm} is the fixed effect of mth year and week of record for kth trait at lth DIM; H_{kln} is the fixed effect of nth herd for kth trait at lth DIM; AAC_{klo} is the fixed effect of oth age at calving for kth trait at lth DIM; D_{ij} is the covariate effect of the DIM of the ith observation for the jth individual; S_j is the random genetic effect of sire of jth individual and e_{ijklmn} is the residual error term for Y.

Figure 1.11 Example of a univariate model fitted to data for milk yield, % fat, % protein and somatic cell count (SCC).

With such large datasets needed for genetic studies, it is essential to be able to use a statistics program to analyse the data e.g. ASREML (Gilmour *et al.*, 2006), SAS (Version 8, SAS Institute Inc., North Carolina, USA), DMU (Madsen & Jensen, 2002). Univariate and multivariate analyses can be run with restricted maximum likelihood (REML). Numerous statistics can be obtained from the analysis packages but the most relevant are the within and between group variances which are used to estimate the heritability. In the above example the variances obtained would be within sires and between sires. The correlation coefficient (t) would be calculated first as the between sire variance divided by the between sire variance plus within sire variance. The correlation coefficient would be multiplied by 4 (because for half sibs $t = ¼ h^2$) to give the heritability (Falconer & Mackay, 1996a). Data analysis is now often much simpler in that a pedigree file (random genetic effect of the animal) can be fitted into the model, using computer software such as ASREML (Gilmour *et al.*, 2006) or DMU (Madsen & Jensen, 2002), which means that the actual variance components are used to calculate the heritability with no need for adjustment.

When estimating heritability the method of estimation is not only determined by the structure of the data available but also in order to minimise bias and maximise precision. Usually the more closely related the individuals the more precise the estimate is because the factor which multiples the regression or correlation coefficient to give the heritability must multiply the standard error of the regression or correlation in order to give the standard error of the heritability (Falconer & Mackay, 1996a). Using second or third degree relatives gives very large standard errors unless the standard error of the regression or correlation was small due to using a very large data set.

In general, bias is either introduced by environmental sources of covariance or by dominance in full sibs (Ponzoni & James, 1978; Falconer & Mackay, 1996a). Estimating heritability by regression of offspring on mother tends to overestimate the parameter because of bias due to maternal effects from the mother such as nutrition during and after pregnancy, health of the mother and weight of the mother (Willham, 1972). Estimating the heritability by regression of offspring on mid-parent can give a slightly less biased result although it is still affected by the maternal effects from the mother. However, a drawback of this method is that the variance of the mothers and fathers must be equal (Falconer & Mackay, 1996a). For the reasons discussed regression of offspring on father and correlation of half sibs gives the most precise and least biased estimates of heritability.

1.7.3 Correlation

Both the phenotypic, genotypic and environmental correlations can be estimated in genetic studies. The phenotypic correlation is the degree to which two traits vary in an individual e.g. tall people tend to be heavier than short people. The phenotypic correlation is due to both genetic and environmental correlations (Lynch & Walsh, 1998a). The phenotypic correlation (r_p) is:

$$r_p = Cov(X,Y) / [Var(X)*Var(Y)]^{½}$$

where

$$Cov(X,Y) = E(X*Y) - \mu_X*\mu_Y$$

and X and Y are two traits.

The environmental correlation is due to two traits being exposed to the same environment and this having an effect on the traits. An environmental correlation can be either positive, e.g. good nutrition can lead to higher weights and heights in individuals, or negative. The environmental correlation is not very easy to directly estimate however if the phenotypic and genetic correlations are known the environmental can be deduced.

The genetic correlation of traits is the correlation of the genetic merit or breeding values for the two traits. The formula for genetic correlation is:

$$r_G(X,Y) = Cov(X,Y) / \sqrt{(Var(X) * Var(Y))}$$

where

$$Cov(X,Y) = [Var(X+Y) - Var(X) - Var(Y)] / 2$$

and X and Y are the breeding values for two traits (Falconer & Mackay, 1996b).

Genetic correlation can arise due to pleiotropy which is one gene affecting more than one trait, due to linkage disequilibrium which occurs when two alleles are close together on the same chromosome, each affects a separate trait and because they are close together they are very likely to be inherited together thus the two traits show correlation (Lynch & Walsh, 1998b). Genetic correlation due to linkage disequilibrium can be reduced over generations due to crossing over of chromosomes during meiosis.

1.8 JUVENILE PREDICTORS OF FEMALE FERTILITY

1.8.1 Previous research

To date there has been little research into the use of juvenile or prepubertal predictors. This is due, in part, to satisfactory mature evaluation, selection being for the traits of highest economic value in the dairy industry and the potential lack of reliability of any juvenile predictor due to unknown correlated response and long term results of selection. A fertility index for dairy cattle has recently been introduced in the UK which will

hopefully improve fertility (Wall *et al.,* 2003a; Section 1.2). However greater rates of genetic improvement in fertility may be possible if a suitable juvenile indicator trait be incorporated into the present fertility index. Land first proposed that sex linked characters in the female are expressed in the male and that reproduction is controlled by the same gonadotrophic hormones in both sexes (Land, 1973). In both male and female cattle, GnRH is released by the hypothalamus which stimulates the anterior pituitary to synthesize and release FSH and LH. In the cow, FSH and LH are important in follicle growth and ovulation (Gong *et al.,* 1996; Milvae *et al.,* 1996; Fortune *et al.,* 2004; Mihm *et al.*, 2002, Section 1.4). In the bull, FSH and LH are important in spermatogenesis (Amann, 1983; Amann & Walker, 1983). In the juvenile heifer and bull LH and FSH initiate and control puberty (Rawlings *et al.*, 2003; Amann, 1983; Section 1.3). For these reasons the hormonal response to GnRH administration has been studied (Royal *et al.*, 2000c).

Selection for fertility in the female sheep is restricted by similar problems faced in dairy cows. Fertility parameters are only measurable in the mature ewe when it first has a lamb and the fertility measures have low heritabilities, e.g. litter size $h^2 = 0.07$, therefore genetic progress is slow (Haley *et al.*, 1989). Haley *et al.* (1989) studied the response of LH to GnRH in the young male sheep with a view to use as an indirect selection criterion for litter size. LH response to GnRH in previous research by Land & Carr (1979), described in Haley *et al.* (1989), has been shown to be highly repeatable and a high response associated with large litter sizes in the Finnish Landrace breed. In the Haley *et al.* (1989) study the LH response to GnRH in 10 week old Finn-Dorset (50% Finnish Landrace and 50% Dorset Horn genes) rams was used to select a high and low response line over eight years. Blood samples were taken 30, 50 and 70 minutes after a 5μg injection of GnRH into the jugular vein. Rams and ewes were used for one and two seasons respectively. The ewes remained in the separate lines but no selection was applied (Haley *et al.*, 1989).

Over the eight years of selection the LH and FSH response to GnRH in the two lines of rams and ewes altered significantly ($P<0.001$) with upto a five fold difference between

the high and low response line (Haley *et al.*, 1989). No significant difference was found in body weight between the two lines. The testes diameter was significantly greater in the high response line at 10 weeks of age but not at 20 weeks of age. Of the female reproductive traits recorded days to first oestrus in the first breeding season, but not in the second breeding season, was significantly shorter in the high response line after eight years of selection (Haley *et al.,* 1989). Ovulation rate was also significantly higher in the high line in the first and second breeding season. Differences in the number of lambs born per ewe were not significant although the high line had a higher average number of lambs on several occasions.

Correlations were calculated as residual correlations after adjustments had been made for the fixed effects. Correlations were found between the various hormone responses, reproductive traits and body weight however they were all mainly small and non significant. A negative significant correlation was found in female lambs between LH response at 20 weeks and days to first oestrus in the second breeding season indicating that a high LH response at 20 weeks may lead to shorter interval to oestrus. In the male lambs significant positive correlations were found between testis diameter at 10 weeks with LH response, FSH response and body weight all at both 10 and 20 weeks of age. Results from this study would indicate that using LH response to GnRH as an indirect selection criterion in the male had no detrimental affect on weight gain or reproductive traits in the male or female. In the ewes, the days to first oestrus in the first breeding season was shorter and the number of lambs per ewe in the high response line was slightly higher than in the low response line. If the effect on litter size was slightly greater, LH response to GnRH in male lambs may be a suitable indirect selection criterion to improve female reproduction.

Mackinnon *et al.* (1991) studied the genetic parameters for testosterone response to GnRH stimulation and the relation to scrotal circumference in tropical beef bulls. Between 500-600 Zebu bulls, weaned at 6 months of age, had liveweight and scrotal circumference measured at 9 and 18 months of age. In the bull scrotal circumference, in addition to sperm production, is an indicator of puberty onset (Lunstra *et al.*, 1978). At

9 and 18 months an injection of GnRH was given followed by a blood sample after 150 minutes. Testosterone concentration was determined by radioimmunoassay. Genetic parameters were estimated by fitting a mixed linear model and using the restricted maximum likelihood method (REML). Heritability estimates for testosterone response and scrotal circumference were moderate at 9 ($h^2 \pm$ standard error, 0.42 ± 0.28, $n = 465$; 0.36 ± 0.12, $n = 707$) and at 18 months of age ($h^2 \pm$ standard error, 0.55 ± 0.15, $n = 501$; 0.28 ± 0.10, $n = 620$; Mackinnon *et al.*, 1991). Testosterone response had a higher heritability and coefficient of genetic variation than scrotal circumference (0.33-0.38 versus 0.08-0.10) indicating that if incorporated into a selection index faster improvement would be seen in testosterone response than scrotal circumference. This study however did not relate testosterone response to GnRH and the scrotal circumference to mature fertility of either the bull or cow which would have given an indication of the possible use of these measures as juvenile predictors of fertility.

A study by Gábor *et al.* (1995) examined the testosterone response to GnRH in Holstein-Friesian bulls and the phenotypic relationship with testes size and sperm quality. Holstein-Friesian bulls ($n = 14$, 5-6 years of age) were injected with a GnRH analogue and jugular blood samples taken just before and 90 minutes after injection. The bulls were slaughtered the day after and testes examined for volume and weight. The results showed a high and significant phenotypic correlation ($r = 0.63$ $P<0.05$, $n = 14$) between testosterone response to GnRH and testes volume (Gábor *et al.*, 1995) thereby suggesting that at a phenotypic level testosterone response to GnRH is a predictor of testes volume (Gábor *et al.*, 1995). Both studies (Gábor *et al.*, 1995; Mackinnon *et al.*, 1991) indicate that testosterone response to GnRH could possibly be used as an indirect selection criterion for testes volume or circumference.

Research has been carried out into indirect selection criteria for milk yield. Selection for milk yield, like fertility, is limited by sex and age. Woolliams and Løvendahl (1991) reviewed previous research into possible predictors of genetic merit for milk yield and highlighted free fatty acids, β-hydroxybutyrate, urea, glucose, thyroxine, GH, IGF-1 and insulin as possible predictors for high milk yield. Due to the negative genetic

relationship between milk yield and fertility possible predictors for milk yield could also be predictors for female fertility.

1.8.2 Potential predictors of female fertility

1.8.2.1 LH and FSH in response to GnRH challenge

As discussed previously (Section 1.6.2) the ultimate predictor for female fertility would be measurable in the male, at an early age, have high heritability and be genetically correlated to female fertility i.e. an indirect selection criterion or a juvenile predictor. Another more practical consideration would be that it should be easy to measure with high accuracy. Ideally this would be a hormone or metabolite that can be measured in plasma or milk. Early work by Royal (Royal, M.D. PhD thesis 1999; Royal *et al.*, 2000c) found that the LH response to GnRH in pre-pubertal (120-140 days) Holstein-Friesian heifers has a high heritability (heritability ± standard error; 0.51 ± 0.29, n = 206) and is correlated to fertility (Royal, M.D. PhD thesis 1999), measured by the commencement of luteal activity postpartum (CLA). If the LH response to GnRH in the prepubertal male has similar heritability and is correlated to female fertility it may prove to be a suitable juvenile predictor. The FSH response to GnRH may also be a possible predictor due to its role in folliculogenesis (reviewed by Webb & Armstrong, 1998) and spermatogenesis (reviewed by Amann, 1983).

1.8.2.2 Testosterone in response to GnRH challenge

The concentration of testosterone in prepubertal male calves may be useful in predicting the future fertility of the bull itself and the fertility of its female offspring. In the adult male, testosterone is synthesised and released by the Leydig cells, in response to LH release from the anterior pituitary, and it maintains spermatogenesis (Amann, 1983). In the bull calf during infancy (birth – 10 weeks), testosterone production in the Leydig cells is minimal due to low concentrations of GnRH and thus LH (Amann *et al.*, 1986).

From 10-20 weeks of age, LH pulsatility increases causing the testes to grow and testosterone production by the Leydig cells increases (Evans *et al.*, 1995).

Previous research (Mackinnon *et al.*, 1991; Gábor *et al.*, 1995; See 1.8.1 for details) has found that testosterone response to GnRH in young bulls is heritable and genetically correlated to testes volume. Furthermore, because the genetic variation in LH response to GnRH is controlled by the same genes in both the male and female (Haley *et al.*, 1989) and testosterone release is dependent on LH release, the testosterone response to GnRH in the male could be correlated to female fertility.

In Holstein bulls, puberty is reached earlier than in tropical beef bulls. Assuming that the heritability and genetic variation seen in the Mackinnon study is partly due to the time of the measurement in relation to puberty onset, this will need to be altered for the Holstein. In the Holstein bull calf, puberty occurs between 9-10 months of age and as LH pulsatility and thus testosterone release increases from 10-20 weeks of age perhaps testosterone response to GnRH at 20 weeks of age would be most useful (Amann, 1983). In practical terms, the earlier the measurement is made the sooner a decision to cull or rear the bull calf, as a result of the measurement, can be made which is an economic advantage. Also at 20 weeks of age the bull calf will be approximately 150 kg in weight making sampling easier than in a larger animal (Amann, 1983).

1.8.2.3 Oestradiol

The concentration of oestradiol in prepubertal male calves may be useful in predicting the future fertility of the bull itself and the fertility of its female offspring. Oestradiol is an important hormone in both male and female reproduction. In the male calf, oestradiol has an inhibitory effect on the hypothalamus which reduces in the prepubertal period to allow LH pulsatility to increase (Amann, 1983; see 1.3 for details). In the adult male, oestradiol is important in maintaining normal spermatogenesis and by binding to receptors in the Leydig cells it is thought to maintain the normal function of the Leydig cells (Amann, 1983; see 1.3 for details). In the prepubertal heifer calf, low

concentrations of oestradiol have a negative effect to prevent LH pulse frequency increasing. In the weeks prior to puberty the sensitivity of the hypothalamus to oestradiol negative feedback reduces allowing LH pulses to increase (Day *et al.*, 1984 & 1987; see 1.3 for details). In the adult cow, oestradiol produced by the growing follicles, has both a positive feedback (follicular phase) and a negative feedback effect (luteal phase) on LH release (Milvae *et al.,* 1996; Mihm *et al.,* 2002; see 1.4 for details).

The important role of oestradiol in controlling LH pulsatility in the prepubertal male and female, the role in maintaining normal spermatogenesis, the production by the dominant follicle, the role in oestrus behaviour and in causing the pre-ovulatory LH surge, all indicate that oestradiol concentration at some point in the juvenile male may predict fertility in the adult female. However, there has been no previous research into the use of oestradiol concentrations in the juvenile as an indirect selection criterion. In addition to this, the heritability, genetic variation and genetic correlation of oestradiol with any other parameters is currently unknown. For these reasons it would be a risk to carry out such research because large studies are needed to confidently estimate genetic parameters and it would take some years to estimate the genetic correlation between prepubertal oestradiol concentrations with adult female fertility.

1.8.2.4 Growth hormone, IGF-1 and insulin

Growth hormone (GH) plays an important role in metabolism, growth and folliculogenesis and therefore may prove a potential predictor of fertility. Growth hormone is released in pulses throughout the day from the anterior pituitary gland in response to growth hormone releasing hormone (GHRH) from the hypothalamus (*Figure 1.12*). The alternating release of GHRH and somatostatin from the hypothalamus controls GH concentration and causes release to be pulsatile.

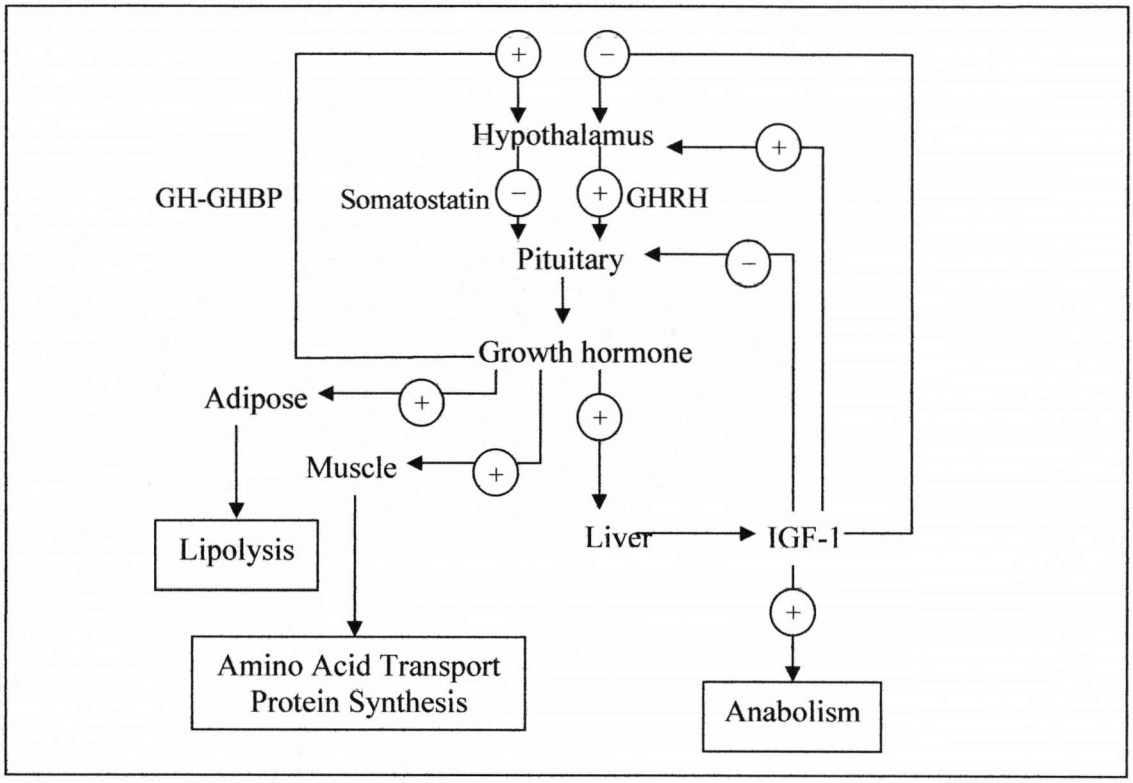

Figure 1.12 Endocrine control of growth hormone release (adapted from Clark & Robinson, 1996).

Growth hormone release is also thought to decrease following feeding and increase during sleep and exercise. Diet and age can affect the release of GH. This was shown in a study by Katoh *et al.* (2004) who investigated changes in post-prandial release of GH in 3 week old milk fed and 12 week old weaned Holstein calves. Milk fed 3 week old calves had higher basal concentrations of GH than 12 week old weaned calves (approximately 12ng/ml versus 8ng/ml). Growth hormone concentrations increased after feeding in the milk fed calves yet fell in the weaned calves. Growth hormone concentration rose also after feeding in 12 week old milk fed calves indicating that the rise is due to diet and stage of development of the rumen rather than age of the calf.

Growth hormones primary function is stimulating growth directly by protein synthesis and indirectly by stimulating the liver to produce IGFs which cause skeletal growth and follicle growth (Langhout *et al.*, 1991; Webb *et al.*, 2004). Growth hormone also

stimulates progesterone and oxytocin synthesis in the large luteal cells of the corpus luteum (Schams & Berisha, 2004).

Insulin is released in a pulsatile manner from the islets of Langerhans in the pancreas (Permutt & Kipnis, 1972). Insulin's main function is regulation of blood glucose concentration in conjunction with glucagons (reviewed by Harmon, 1992; Randle & Ashcroft, 1969). However insulin is important in folliculogenesis in that it stimulates steroid production by the growing follicle, granulosa cell proliferation, and it stimulates progesterone production by the corpus luteum (Langhout *et al.*, 1991; Mihm *et al.*, 2002; Webb *et al.*, 2004).

The IGF family are important in many aspects of female reproduction (See 1.3 & 1.4). Gene expression for all of the IGF family has been found in the ovary (Webb & Armstrong, 1998). IGF-1 is of particular importance in that it stimulates follicle growth, oestradiol synthesis by the growing follicle and progesterone synthesis by the corpus luteum (Webb *et al.*, 2002; Fortune *et al.*, 2004). The IGF system would also appear to be important in the male calf as the initiation of puberty has been associated with changes in the IGF system (See 1.3 for details). At puberty some studies have found an increase in IGF-1, an increase in IGFBP-3 and a decrease in IGFBP-2 (Renaville *et al.*, 1996). The association between differing plasma IGF-1 concentrations with conception rate in primiparous and multiparous Holstein-Friesian cows was shown by Taylor *et al.* (2004a). This research found that plasma IGF-1 concentrations were higher in primiparous (n = 142) than multiparous (n = 177) cows. One week postpartum multiparous cows with plasma IGF-1 higher than 25ng/ml were 11 times more likely to conceive than cows with lower IGF-1 concentrations. This finding may indicate that higher concentrations of IGF-1 after calving stimulate increased follicular growth.

Growth hormone, insulin and IGF-1 link the nutritional status of the animal to its fertility. This is particularly evident in the period of NEB postpartum (See 1.4.3.1). During the period of NEB, insulin and glucose concentrations fall, GH concentration increases, yet GH receptor concentrations in the liver decrease causing IGF-1 production

to fall (Webb *et al.*, 1999). The decreased amount of IGF-1 decreases the rate of follicle growth and LH pulsatility reduces (Butler, 2000). This may lead to a smaller dominant follicle which produces less oestradiol and is therefore less able to exert a positive feedback affect on LH and cause the pre-ovulatory LH surge (Mihm, 2002).

Previous research has found that GH appears to be heritable in calves; with heritability estimates higher when GH is measured following a hormone challenge such as GHRH (Grochowska *et al.,* 2001; Løvendahl & Sørenson, 2001; Sørensen *et al.,* 2002). However, results vary from those that found little genetic variation to those that found high heritability. These differences could be due to the different breeds, ages, number of animals and experimental design. Few studies have estimated the heritability of insulin and IGF-1 but there is some evidence that they appear to show genetic variation in calves (heritability range, 0.15 to 0.35; Grochowska *et al.,* 2001; Løvendahl & Sørenson, 2001).

Treatment of cows during the lactation period with recombinant growth hormone (rGH) is practiced in some countries e.g. USA (Bauman, 1992) to increase milk production. However, other effects on reproduction have been found. Treatment with rGH was shown to increase twinning rate from 5 to 10% in two studies (Cole *et al.,* 1991, Wilkinson and Tarrant, 1991) which in cattle is not advantageous. Gong *et al.,* (1991) gave beef heifers twice-daily injections of rGH and found that this significantly increased the number of follicles between 2-5mm in diameter. A further study by Gong *et al.,* (1996) found that treatment of ewes with rGH significantly increased peripheral concentration of GH ($P<0.01$), IGF-1 ($P<0.01$), insulin ($P<0.01$) and progesterone ($P<0.05$) whilst concentration of FSH and LH was unaffected. Control and treated ewes had the same number of follicles $\geq$1mm in diameter. Treatment with rGH significantly increased the number of medium sized follicles (2-4mm diameter). When removed and incubated, large and small follicles from rGH treated ewes secreted significantly more IGF-1 than control follicles. This paper would indicate that treatment with rGH has a beneficial effect on follicle and peripheral concentrations of GH, insulin, progesterone and IGF-1 which may in turn enhance follicle growth (Gong *et al.*, 1996).

1.8.2.5 Glucose and free fatty acids

Glucose concentrations remain very stable in cattle and in other ruminants. During periods of dietary restriction, lowered plasma glucose concentration reduces LH pulsatility through GnRH (Diskin *et al.*, 2003). A reduction in LH pulsatility and a reduced ability of the dominant follicle to produce a pre-ovulatory LH surge during NEB are one of the main factors leading to reduced fertility. It would appear that glucose may link metabolic status with fertility (Diskin *et al.*, 2003).

Lipolysis of stored fat produces free fatty acids (FFA) and glycerol. These are released into the blood stream where they are transported to tissue for use as an energy source. Concentrations of FFA increase during periods of energy shortage such as NEB (Bobe *et al.*, 2004). The concentration of FFA in the blood is a good indicator of the metabolic status of an animal. Although FFA are not believed to play any direct role in reproduction they are elevated during metabolic stress caused by energy restriction. An ability to withstand the high demands of milk production in early lactation and suffer less severe NEB may result in fewer fertility problems. For these reasons lower concentrations of FFA following a fast could be an indication of an ability to cope with NEB and therefore FFA may be a suitable juvenile predictor of fertility.

There have been few studies investigating genetic variation in concentrations of FFA and glucose in prepubertal calves. Rowlands *et al.* (1983) found glucose to be heritable in fed British Friesian bulls (n = 428; 3-15 months of age; $h^2 = 0.41 \pm 0.17$). Similarly, Løvendahl and Jensen (1997) found glucose and FFA, in addition to insulin, GH and urea, to be heritable in male and female calves (n = 800 total, 9 months of age, heritability range, 0.22 to 0.52).

1.9 THESIS OBJECTIVES

Although previous research has found genetic variation in concentrations of FFA, glucose, GH, insulin and IGF-1, results are varied and generally in older animals (9 months to 12 months). Furthermore, the importance of these hormones and metabolites in reproduction and metabolic regulation points towards the possibility of a genetic relationship between concentrations in the calf and subsequent adult female fertility which to the authors' knowledge has not been studied. Therefore the objectives of this thesis are to assess whether genetic variation is present in the concentrations of FFA, glucose, GH, insulin and IGF-1 concentrations in pre-pubertal calves and secondly to assess whether these concentrations are correlated, both genetically and phenotypically, to female fertility traits and other traits of economic importance.

Chapter 2: MATERIALS AND METHODS

This chapter describes the laboratory procedures and assay statistics utilised for blood and milk sample analysis. Detailed descriptions of the datasets used and sample collection is described in detail in Chapter 3 and will be referred to where necessary.

2.1 MILK PROGESTERONE MEASUREMENT

The progesterone concentration of individual milk samples was determined by a milk progesterone enzyme linked immuno-sorbent assay kit (ELISA; Ridgeway Science Ltd., Alvington, Glocestershire, UK) based on the method by Sauer *et al.* (1986). All reagents and plates were obtained from Ridgeway Science unless otherwise stated. Assays were completed by the University of Nottingham (Division of Animal Physiology, University of Nottingham, Sutton Bonington Campus, Loughborough, Leics, LE12 5RD, UK).

2.1.1 Calibrators and quality control

Two quality control samples were used; these were 2 ng/ml and 8 ng/ml progesterone in milk. Seven calibrators were used to give a standard curve (0, 1, 2, 5, 10, 20 and 50 ng/ml progesterone).

2.1.2 Assay buffer

Assay buffer sufficient for one plate was made by dissolving the substrate tablets in 25 ml of substrate buffer 2, both provided in the assay kit. These were mixed and left to dissolve 20-30 min.

2.1.3 Assay protocol

Microtitre plates, coated with bovine anti-progesterone, and milk samples (30 ml in plastic milk pots; Massmould, Luton, UK) each with one Lactab Mark 3 milk preservative tablet (Thompson & Capper Ltd., Runcorn, UK), both stored at 4 °C until use, were brought upto room temperature (20-22 °C). Whole milk samples were vortexed thoroughly. The foil covering was stripped off the plates, the wells emptied and tapped dry. To each well, 10 µl of standard (triplicate), quality control (duplicate) or sample (duplicate) was added. Progesterone-enzyme label (200 µl per well) was then added and incubated at room temperature for 80 min, to allow the progesterone in the milk samples, quality controls and standards to compete with the bovine anti-progesterone for the progesterone enzyme label. Plate wells were then washed out three times with distilled water and tapped dry each time. Substrate in substrate buffer (200 µl) was then added to each well. The plates were further incubated for 25 min at room temperature and the colour density then read at 570 nm on an automatic plate reader (Titertek Multiskan MAC/340).

Milk progesterone assays were carried out between August 2004-April 2006 and were analysed in 13 batches with varying number of plates per batch (one plate per 34 milk samples).

2.1.4 Reliability Criteria

A representative standard curve is given in *Figure 2.1*. This is the mean standard curve of the 13 batches (calculated from 10 randomly selected plates from the total 196 plates used). Estimated dose (ED) values at 20, 50 and 80 % absorbance on the standard curve were also monitored to ensure the assay was reproducible (See *Table 2.1 & 2.2*). From *Tables 2.1 & 2.1* it can be seen that the ELISA remained stable over the period of use.

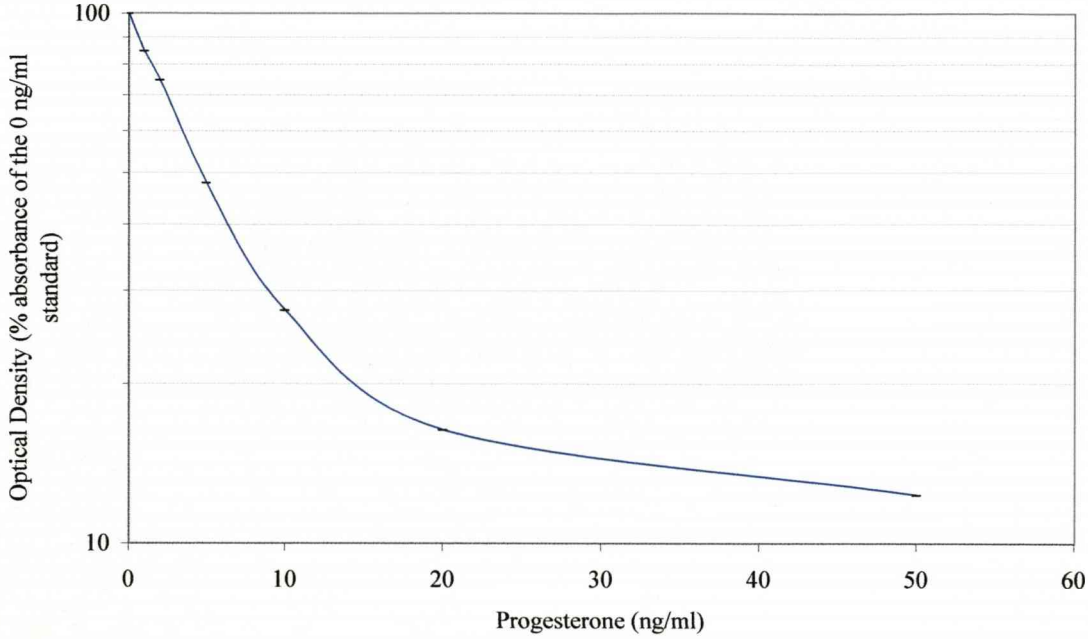

Figure 2.1 Representative standard curve (average data from 10 randomly selected plates from the total 196 plates used) for the progesterone assay showing the optical density as a proportion of the absorbance of the 0 ng/ml standard plus standard errors (standard errors shown are small therefore can not be seen clearly).

Table 2.1 Estimated dose (ED) values at 20, 50 and 80 % absorbance for the milk progesterone ELISA (one randomly chosen assay from each batch).

Batch No	**Number of plates per batch**	**ED at 20 % absorbance (ng/ml)**	**ED at 50 % absorbance (ng/ml)**	**ED at 80 % absorbance (ng/ml)**
1	5	2.05	5.04	9.73
2	7	1.86	5.09	9.38
3	10	2.30	4.60	10.91
4	10	2.20	4.75	10.76
5	17	1.96	4.95	10.29
6	29	2.42	4.71	10.60
7	20	1.98	4.79	10.82
8	32	1.76	4.96	10.28
9	10	1.58	4.71	10.70
10	15	2.03	4.96	10.62
11	10	2.20	4.83	10.40
12	10	1.83	5.33	10.32
13	21	2.09	5.00	9.14

Table 2.2 Descriptive statistics for the progesterone assay

Statistic	**ED at 20 % absorbance (ng/ml)**	**ED at 50 % absorbance (ng/ml)**	**ED at 80 % absorbance (ng/ml)**
Mean	2.02	4.90	10.30
Standard deviation	0.23	0.20	0.56
Standard error	0.06	0.05	0.15
Coefficient of variation	11.41	4.00	5.41

2.1.5 Sensitivity

The working range of the assay was 1-23.5 ng/ml (Royal, 1999). The sensitivity of the assay is the minimum amount that the assay can detect or that it can distinguish between two samples with similar concentrations. The sensitivity of this assay was taken as the lowest standard (1 ng/ml).

2.1.6 Precision

The intra-assay coefficients of variation for the 2 and 8 ng/ml quality controls were 7.69 % and 4.20 % respectively (calculated using a representative 50 assays). The inter-assay coefficients of variation for the 2 and 8 ng/ml quality controls were 15.87 % and 10.18 % respectively (calculated using a representative 50 assays).

All results above 20 ng/ml progesterone were re-assayed using half of the amount of milk in each well i.e. 5 µl to give a more accurate measurement for samples with higher progesterone content. Samples were re-assayed if they had an intra-assay coefficient of variation >15 %.

2.2 PLASMA SAMPLE ANALYSIS

Analysis of all plasma samples (for growth hormone, insulin, insulin like growth factor 1, free fatty acids and glucose) was carried out at the Danish Institute of Agricultural Sciences (Department of Genetics and Biotechnology and the Department of Animal Health, Welfare and Nutrition, Danish Institute of Agricultural Sciences, Research Centre Foulum, Blichers Allé, Postbox 50, DK-8830, Tjele, Denmark).

2.2.1 Growth hormone

The bovine growth hormone (GH) content was determined by a validated noncompetitive time-resolved immunofluorometric assay, run semi-automated on a

time-resolved fluorometer (Autodelfia, Wallac Oy, Turku, Finland), as previously described by Løvendahl *et al.* (2003).

2.2.1.1 Calibrators and quality control

Antibodies were raised against recombinant bovine GH (rbGH, Danish Veterinary Serum Institute, Copenhagen). The rbGH was used to prepare calibrators in heat inactivated serum (0.20, 0.40, 1.00, 4.00, 40, 100 & 200 ng/ml; 56 ± 1°C for 3 hours; filtered through a glass filter; centrifuged x 4000 g, 4°C, 30 min). A standard solution of 10 µg/ml rbGH in serum was stored at -20 °C until needed. Low, medium and high GH quality controls were used. Low GH plasma was obtained from a lactating cow, medium GH from a bull calf following i.v. stimulation with synthetic GHRH (Bachem, Feinchemichalien AG, Bubendorf, Switzerland) and high GH from a calf following i.v. stimulation with GHRH and thyrotropin-releasing hormone (Bachem).

2.2.1.2 Monoclonal antibodies

Splenocytes, a type of monocyte found in the spleen, where removed from Balb/c mice that had been injected three times at two week intervals with rbGH. The splenocytes were fused with myeloma cells (Danish Veterinary Serum Institute, Copenhagen) to give hybridomas. Hybridoma clones which were showing the highest level of immunoreactivity were recloned and propagated to produce a matching pair of antibodies. One (mab1.15) was used for coating the microtitre plates and one (mab1.2) for the Eu^{3+} labeled detection antibody.

2.2.1.3 Assay buffer

The assay buffer was made up of 100 mmol/l Tris-HCl, 150 mmol/l NaCl (Sigma-Aldrich, Germany), 0.05 % NaN_3(Sigma-Aldrich, Germany), 20 µmol/l diethylene triamine penta-acetic acid (Sigma-Aldrich, Germany), 0.5 % BSA (A1662; Sigma-

Aldrich, Germany), and 0.1 % Tween-20 (Merck Schuchart GmbH, Hohenbrunn, Germany).

2.2.1.4 Coating microtitre plates

Microtitre plates (Delfia Plates, Wallac Oy, Turku, Finland) were coated with 200 µl per well of the coating antibody solution (100 m*M* NaH_2PO_4; Merck, Darmstadt, Germany) pH 4.90) which contained 680 ng/200µl of the antibody mab1.15. Following incubation (4 °C, overnight) plates were washed once with wash solution (Wallac Oy, Turku, Finland) and blocked with 250 µl/well of a liquid containing wash solution plus 0.5 % Tween-20 (Merck Schuchart GmbH, Hohenbrunn, Germany). The microtitre plate was then gently shaken for at least one hour and then sealed and stored at 4 °C.

2.2.1.5 Eu^{3+} labelling of detection antibody

Antibody mab1.2 was labelled with Eu^{3+} according to the manufacturers' instructions for kit 1244-302 (Delfia, Wallac Oy, Turku, Finland).

2.2.1.6 Assay protocol

Microtitre plates were washed once with wash solution (Wallac Oy, Turku, Finland) then 50 µl of calibrator (duplicate), quality control (duplicate) or sample (single) was added to the plate wells. Then 200 µl of assay buffer containing 25 ng/well of mab1.2 was added. Followed by incubation at room temperature for 4 hours with slow shaking. Plates were then washed 4 times with wash solution (Wallac Oy, Turku, Finland). Enhancement solution (200 µl/well; Wallac Oy, Turku, Finland) was added and plates were shaken for 10 min. The enhancement solution dissociates Eu^{3+} from solid phase bound Eu labelled antibodies in a few minutes to form a homogenous and highly fluorescent solution which allows highly sensitive Eu^{3+} measurements to be made (Wallac Oy, Turku, Finland). Fluorescence was then measured on a time-resolved

fluorometer (Autodelfia, Wallac Oy, Turku, Finland). A smoothing spline algorithm on log-log values fitted the standard curve.

2.2.1.7 Reliability criteria

A representative standard curve for growth hormone is shown in *Figure 2.2*. This is the mean standard curve of the 17 batches (one randomly selected plate taken from each of the 17 batches). Estimated dose values at 20, 50 and 80 % absorbance on the standard curve were also monitored to ensure the assay was reproducible (See *Table 2.3 & Table 2.4*). From *Table 2.3 & 2.4* it can be seen that the assay remained stable over the period of use.

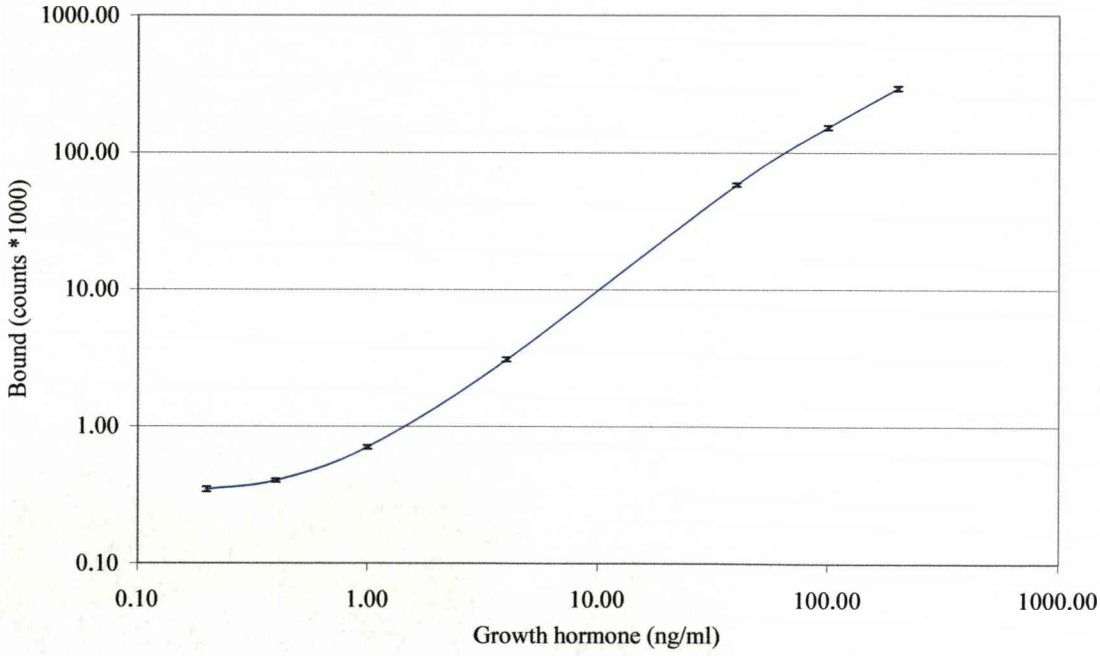

Figure 2.2 Representative standard curve for the growth hormone assay showing the average data from the 17 batches plus the standard error.

Table 2.3 Estimated dose (ED) values at 20, 50 and 80 % absorbance for the growth hormone assay (one randomly chosen plate from each batch).

Batch No	**Number of plates per batch**	**ED at 20 % absorbance (ng/ml)**	**ED at 50 % absorbance (ng/ml)**	**ED at 80 % absorbance (ng/ml)**
1	1	1.79	9.32	47.65
2	1	1.65	8.89	44.55
3	2	1.68	8.84	50.21
4	3	1.71	8.85	47.28
5	3	1.87	9.45	43.61
6	3	1.75	9.44	49.23
7	1	1.66	9.32	48.36
8	2	1.71	8.85	47.28
9	1	1.75	9.44	49.23
10	2	1.75	9.44	49.23
11	1	2.04	11.44	63.44
12	2	1.98	11.36	56.19
13	2	2.24	10.54	51.68
14	2	2.16	10.73	56.50
15	2	2.22	11.23	56.78
16	2	1.82	10.35	52.92
17	1	2.19	12.05	54.08

Table 2.4 Descriptive statistics for the growth hormone assay

Statistic	**ED at 20 % absorbance (ng/ml)**	**ED at 50 % absorbance (ng/ml)**	**ED at 80 % absorbance (ng/ml)**
Mean	1.88	9.97	51.07
Standard deviation	0.21	1.06	5.07
Standard error	0.05	0.26	1.23
Coefficient of variation	11.23	10.62	9.92

2.2.1.8 Sensitivity

The working range of the assay was 0.2 – 200 ng/ml (Løvendahl *et al.*, 2003). The sensitivity (See 2.1.5 for definition) of the assay was taken as the lowest standard (0.2 ng/ml).

2.2.1.9 Precision

The intra-assay coefficients of variation for the low, medium and high quality controls were 6.84 %, 5.33 % and 6.81 % respectively (calculated using all assay data). The inter-assay coefficients of variation across the 17 batches for the low, medium and high quality controls were 15.22 %, 17.68 % and 15.88 % respectively.

Standard curve and quality control data was monitored throughout to ensure results from each plate were correct and in addition individual sample results (single) were inspected following each assay and re-assayed if necessary for example if a clot was detected or if the level of sample in the tube was too low.

2.2.2 Insulin

The bovine insulin content was determined by a validated noncompetitive time-resolved immunofluorometric assay, run semi-automated on a time-resolved fluorometer (Autodelfia, Wallac Oy, Turku, Finland), as previously described by Løvendahl and Purup (2001).

2.2.2.1 Calibrators and quality control

Calibrators were prepared in heat inactivated serum (3.34, 6.68, 13.36, 26.72, 106.90, 427.50 & 1710.00 pmol/l; 56 ± 1 °C for 3 hours; filtered through a glass filter; centrifuged x 4000 g, 4 °C, 30 min) using monocomponent bovine insulin with biological activity of 26.9 IU/mg (Novo Nordisk Farmaka, Novo Allé, 2880 Bagsværd,

Denmark). Plasma from calves was used to prepare quality controls. Low insulin plasma was obtained from overnight starvation, medium from *ad libitum* feeding, and high obtained after administration of an intravenous bolus injection of glucose (500 g/l Sygehus Apotekerne, Copenhagen, Denmark). Quality controls and calibrators were stored at -20 °C until needed.

2.2.2.2 Monoclonal antibodies

Paired monoclonal antibodies were raised against human insulin (Dako UK Ltd., Denmark House, Angel Drove, Ely, Cambridgeshire, UK) and against bovine insulin. Plates were purchased that had been coated with antibody and were ready to use (Wallac Oy, Turku, Finland).

2.2.2.3 Assay buffer

The assay buffer was made up of 100 mmol/l Tris-HCl, 150 mmol/l NaCl (Sigma-Aldrich, Germany), 0.05 % NaN_3 (Sigma-Aldrich, Germany), 20 µmol/l diethylene triamine penta-acetic acid (Sigma-Aldrich, Germany), 0.5 % BSA (A1662; Sigma-Aldrich, Germany), and 0.1 % Tween-20 (Merck Schuchart GmbH, Hohenbrunn, Germany).

2.2.2.4 Assay protocol

Microtitre plates were washed once with wash solution (Delfia Plates, Wallac Oy, Turku, Finland) then 50 µl of calibrator (duplicate), quality control (duplicate) or sample (single) was added to plate wells. Then 200 µl of assay buffer containing 25 ng/well of Eu^{3+} labelled antibody was added (Wallac Oy, Turku, Finland). Followed by incubation at room temperature for 4 hours with slow shaking. Plates were then washed 4 times with wash solution (Wallac Oy, Turku, Finland). Enhancement solution (200 µl/well) (Wallac Oy, Turku, Finland) was added and plates were shaken for 10 minutes. The enhancement solution dissociates Eu^{3+} from solid phase bound Eu labeled antibodies in

a few minutes to form a homogenous and highly fluorescent solution which allows highly sensitive Eu^{3+} measurements to be made (Wallac Oy, Turku, Finland). Fluorescence was then measured on a time-resolved fluorometer (Autodelfia, Wallac Oy, Turku, Finland). A smoothing spline algorithm on log-log values fitted the standard curve.

2.2.2.5 Reliability criteria

A representative standard curve for insulin is shown in *Figure 2.3*. This is the mean standard curve of the 17 batches (one randomly selected plate taken from each of the 17 batches). Estimated dose values at 20, 50 and 80 % absorbance on the standard curve were also monitored to ensure the assay was reproducible (See *Table 2.5 & Table 2.6*). From *Tables 2.5 & 2.6* the assay appeared to remain stable over the period of use.

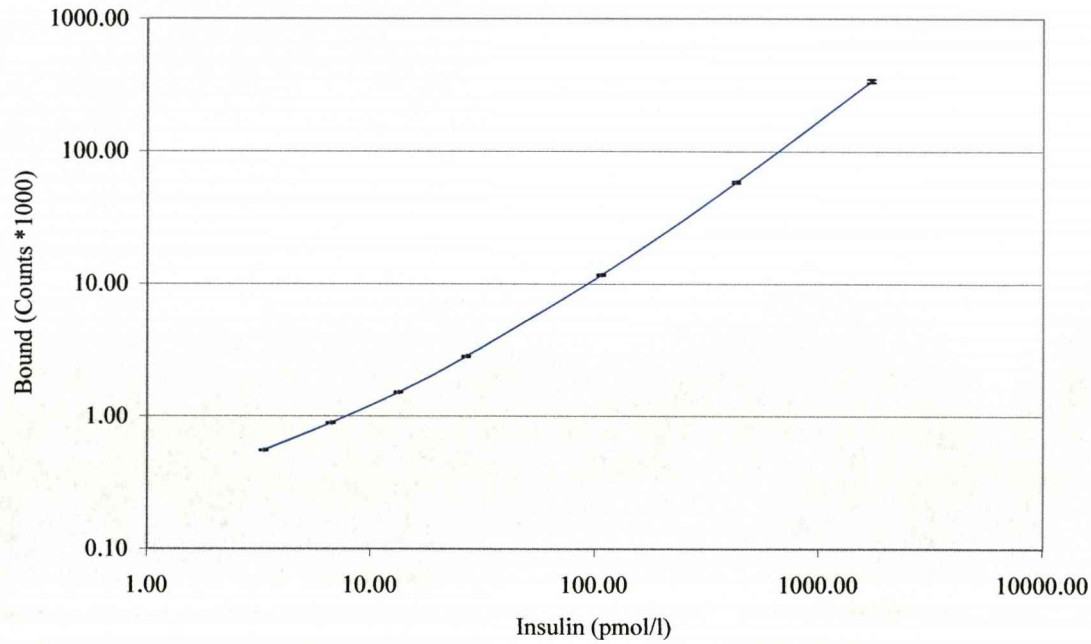

Figure 2.3 Representative standard curve for the insulin assay showing the average data from the 17 batches and the standard error.

Table 2.5 Estimated dose (ED) values at 20, 50 and 80 % absorbance for the growth hormone assay (one randomly chosen plate from each batch).

Batch No	**Number of plates**	**ED at 20 % absorbance (pmol/l)**	**ED at 50 % absorbance (pmol/l)**	**ED at 80 % absorbance (pmol/l)**
1	1	17.20	108.2	577.6
2	1	16.51	110.3	590.8
3	2	17.46	115.2	588.1
4	3	19.47	136.3	672.4
5	3	18.68	127.9	652.2
6	3	18.67	129	652.2
7	1	18.93	118.6	587.8
8	2	18.45	128.2	629.2
9	1	18.92	129.9	653.1
10	2	18.52	125.8	630.5
11	1	17.46	125.2	630.9
12	2	18.36	126.6	632
13	2	17.48	112.8	585.5
14	2	18.31	123.6	631.3
15	2	17.83	128.3	646.8
16	2	18.82	128.5	639.3
17	1	18.87	123.8	627.9

Table 2.6 Descriptive statistics for the insulin assay

Statistic	**ED at 20 % absorbance (pmol/l)**	**ED at 50 % absorbance (pmol/l)**	**ED at 80 % absorbance (pmol/l)**
Mean	18.23	123.42	625.15
Standard deviation	0.78	7.74	28.61
Standard error	0.19	1.88	6.94
Coefficient of variation	4.28	6.27	4.58

However monitoring of the low, medium and high quality control samples for each plate showed that 6 of the batches had within plate drift. The drift was evident in that the insulin concentration in the quality control samples fell from the start to the end of the plate. Whether a plate significantly drifted was determined by fitting a mixed model to the low, medium and high quality control results. The position number (that is whether the result was at the beginning or end of the plate) and the replicate number (1 or 2) were fitted into the model as fixed effects. The assay number and plate number interaction nested within position number was fitted as a random effect. Individual adjustment factors (average plus standard deviation, 1.14 ± 0.15) were calculated for all samples in the six assays with drift (six assays with a total of 14 plates) and insulin level altered. The objectives of the adjustments made were to remove the systematic drift part of the variation whereas variance and mean were not changed. The adjustment factors were calculated using a computer programme that took into account the position of the sample on the plate, the amount of drift in the plate and the counted fluorescence (concentration of insulin) of the sample.

2.2.2.6 Sensitivity

The working range of the assay was 3-16700 pmol/l, the upper limit being much higher than any samples in this study (Løvendahl *et al.*, 2002). See 2.1.5 for sensitivity definition. The sensitivity of the assay was taken as the lowest standard (3 pmol/l).

2.2.2.7 Precision

The intra-assay coefficients of variation for the low, medium and high quality controls were 5.67 %, 3.48 % and 2.04 % respectively (calculated using all assay data after adjusting for drift, See 2.2.2.5 for details). The inter-assay coefficients of variation across the 17 batches for the low, medium and high quality controls were 6.22 %, 4.10 % and 4.06 % respectively (calculated after adjustment for drift).

Standard curve and quality control data was monitored throughout to ensure results from each plate were correct and in addition individual sample results (single) were inspected following each assay and re-assayed if necessary for example if a clot was detected or if the level of sample in the tube was too low.

2.2.3 Insulin like growth factor 1

The total bovine insulin like growth factor 1 (IGF-I) content was determined by a noncompetitive time-resolved immunofluorometric assay based on an assay previously described by Frystyk *et al.* (1995).

2.2.3.1 Calibrators and quality control

Synthetic IGF-I (Roche, Switzerland) 10 µg was diluted in assay buffer and aliquots of 1ml containing 10 µg/l were stored at -20 °C until needed. Seven dilutions were used as calibrators and made fresh each time (5, 10, 20, 78, 312, 625 & 1250 ng/ml). Plasma from cows was used to prepare quality controls. High IGF-1 was obtained from an overnight fasted cow and low IGF-I from a fed cow (stored at -20 °C).

2.2.3.2 Monoclonal antibodies

Two monoclonal antibodies were used; MAB 41 (Novo Nordisk A/S, Bagsvaerd, Denmark) and antibody clone 21 (Kit 5600, Diagnostic System Laboratories Inc.,

Webster, TX, USA). One (MAB 41) was used for coating the microtitre plates and one (antibody clone 21) for the Eu^{3+} labeled detection antibody.

2.2.3.3 Eu^{3+} labelling of detection antibody

Antibody clone 21 was labelled with Eu^{3+} according to manufacturer instructions (Wallac Oy, Turku, Finland).

2.2.3.4 Assay buffer

The assay buffer was made up of 200 mmol/l Tris, pH 8.0, 2 g/l chicken albumin, 6 g/l NaCl (Sigma-Aldrich, Germany), 0.1 % Tween-20 (Merck Schuchart GmbH, Hohenbrunn, Germany), 1.6 g/l Titriplex V (diethylene triamine penta acetic acid, DTPA; Sigma-Aldrich, Germany) and 0.05 % NaN_3 (Sigma-Aldrich, Germany).

2.2.3.5 Coating of microtitre plates

Microtitre plates (Wallac Oy, Turku, Finland) were coated with 250 µl per well of the coating antibody solution (15 mmol/l sodium carbonate, 35 mmol/l sodium hydrogen carbonate, pH 9.6) which contained 2.5 µg/ml of antibody MAB 41. The plates were left overnight (37 °C) then washed once with wash solution (Wallac Oy, Turku, Finland) and blocked with 300 µl per well blocking buffer (4 g/l chicken albumin, Sigma-Aldrich, Germany dissolved in 40 mmol/l phosphate, 0.6 mmol/l sodium merthiolate, pH 8.0, 6 g/l NaCl, and 1.6 g/l Titriplex V (diethylene triamine penta acetic acid, DTPA; Sigma-Aldrich, Germany)).

2.2.3.6 Serum extraction

750 µl of ethanol was put in eppendorf tubes and placed on ice. To this 30 µl of plasma was added and immediately vortexed followed by 2 hours incubation on ice (4 °C).

After this time they were centrifuged (x 2500 g, 4 °C, 15 min). 15 µl of supernatant was added to 600 µl of assay buffer and vortexed and used immediately in the assay.

2.2.3.7 Assay protocol

The microtitre plates were washed once with wash solution (Wallac Oy, Turku, Finland) then 200 µl of calibrator, quality control or sample were added to wells in duplicate. The plates are incubated overnight, at room temperature, in a polystyrene box. The plates were washed once with wash solution (Wallac Oy, Turku, Finland). Then add 200 µl of Eu^{3+} labelled antibody. Followed by incubation at room temperature for 2 hours. Plates were then washed six times with wash solution (Wallac Oy, Turku, Finland). Enhancement solution (200 µl/well; Wallac Oy, Turku, Finland) was added to detach bound $Eu^{3+}$and plates were shaken for 5 min. Fluorescence was then measured on a time-resolved fluorometer (DELFIA, Wallac Oy, Turku, Finland).

2.2.3.8 Reliability criteria

A representative standard curve for IGF-I is given in *Figure 2.4*. This is the average standard curve from the 32 plates. Estimated dose values at 20, 50 and 80 % absorbance on the standard curve were also monitored to ensure the assay was reproducible (See *Table 2.7 & Table 2.8*). It is evident from *Table 2.7 & 2.8* that the assay remained stable over the period of use.

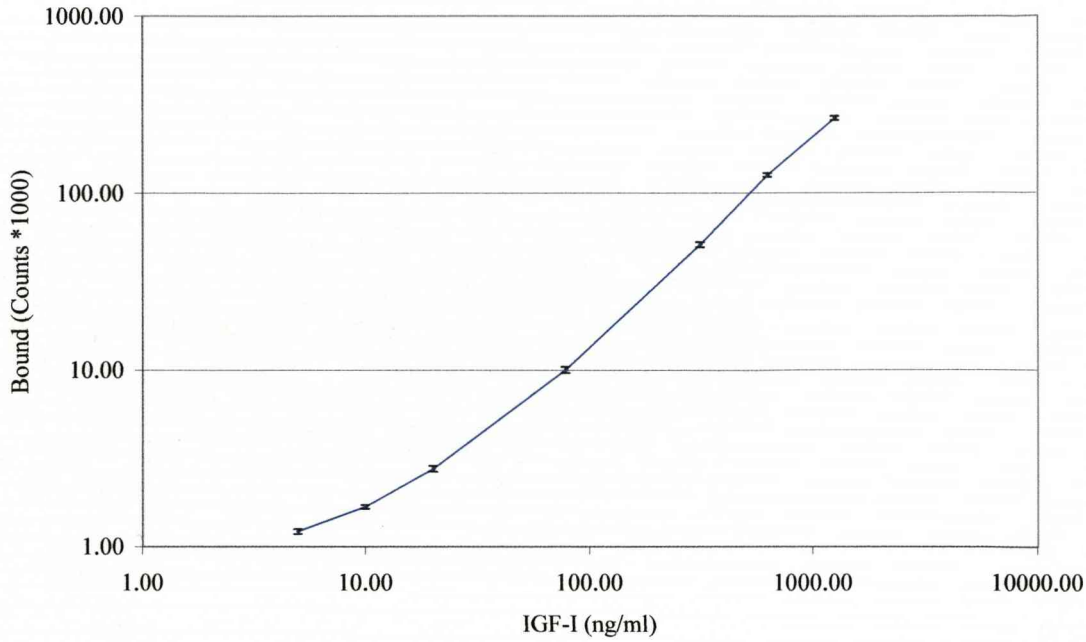

Figure 2.4 Representative standard curve for the IGF-I assay showing the average data from the 32 plates and the standard error.

Table 2.7 Estimated dose (ED) values for each plate at 20, 50 and 80 % absorbance for the IGF-I assay.

Plate	ED at 20 % absorbance (ng/ml)	ED at 50 % absorbance (ng/ml)	ED at 80 % absorbance (ng/ml)
1	24.4	112	456
2	24.3	117	462
3	30.2	136	477
4	26.7	121	490
5	25.6	127	478
6	29.0	143	502
7	30.3	139	500
8	28.3	140	449
9	30.9	142	484
10	30.7	158	512
11	29.6	135	492
12	24.6	132	469
13	27.1	123	493
14	26.3	121	472
15	24.2	127	410
16	25.4	126	467
17	23.4	119	475
18	26.6	136	483
19	25.1	129	481
20	23.7	112	462
21	25.6	136	496
22	33.6	143	519
23	28.5	150	505
24	30.0	159	459
25	27.0	138	490
26	29.8	146	515

27	26.0	141	526
28	25.0	118	480
29	24.3	131	474
30	23.0	124	504
31	24.9	134	475
32	25.1	140	521

Table 2.8 Descriptive statistics for the IGF-I assay.

Statistic	**ED at 20 % absorbance (ng/ml)**	**ED at 50 % absorbance (ng/ml)**	**ED at 80 % absorbance (ng/ml)**
Mean	26.85	132.97	483.69
Standard deviation	2.68	12.00	24.09
Standard error	0.47	2.12	4.26
Coefficient of variation	9.99	9.03	4.98

2.2.3.9 Sensitivity

The working range of the assay is 5-2500 ng/ml, the top end being much higher than any samples in this study (Frystyk *et al.*, 1995). See 2.1.5 for sensitivity definition. The sensitivity of the assay was taken as the lowest standard (5 ng/ml).

2.2.3.10 Precision

The intra-assay coefficients of variation for the low and high quality controls were 9.08 % and 22.98 % respectively (calculated using all assay data). The inter-assay coefficients of variation across the 32 plates for the low and high quality controls were 20.37 % and 25.93 % respectively. All results with an intra-assay coefficient of variation above 15 % were re-assayed.

2.2.4 Free fatty acids

Free fatty acid (FFA), also termed non-esterified fatty acid (NEFA), content was determined using a commercial assay kit (NEFA C; Wako Chemicals GmbH, Neuss, Germany) adapted to automated running on a Advia 1650 Bayer Opera system (Bayer, North Carolina, USA).

2.2.4.1 Reagents

Reagents were obtained and made up according to manufacturer instructions (*Table 2.9*).

Table 2.9 Reagents provided with the NEFA C assay kit (Wako Chemicals GmbH)

Item	**Components**	**Concentration**
R1: 1 bottle	Solvent A	65 mL
	Phosphate Buffer, pH 6.9	50 mmol/L
	Magnesium chloride	3.0 mmol/L
	Surfactant	
	Stabilizers	
R1a: 6 bottles	Color Reagent A	For 10mL
	ACS (Acyl-CoA-Synthetase)	0.3 kU/L
	AOD (Ascorbate oxidase)	3.0 kU/L
	CoA (Coenzyme A)	0.6 g/L
	ATP (Adenosine triphosphate)	5.0 mmol/L
	4-Aminophenazone	1.5 mmol/L
R2: 1 bottle	Solvent B	130 mL
	MEHA	1.2 mmol/L
	(3-Methyl-N-Ethyl-N-(β-hydroxyethyl)aniline)	
	Surfactant	
R2a: 6 bottles	Color Reagent B	For 20 mL
	ACOD (Acyl-CoA-Oxidase)	6.6 kU/L
	POD (Peroxidase)	7.5 kU/L

Reagent A and B were prepared according to instructions with the kit solutions (*Table 2.10*).

Table 2.10 Preparation of reagent A and reagent B (Wako Chemicals GmbH, Neuss, Germany)

Reagent	**Instructions**
Reagent A	Dissolve contents of bottle R1a with 10 mL R1 and mix well. Store at 2-10°C and use within 5 days.
Reagent B	Dissolve contents of bottle R2a with 20 mLR2 and mix well. Store at 2-10°C and use within 5 days.

2.2.4.2 Calibrators and quality control

A FFA standard solution was obtained from Wako for calibration. Six dilutions were used as calibrators and made fresh each time (0, 100, 300, 500, 700 & 100 μeqv/l). Plasma from cows was used to prepare a low and high FFA quality control containing 480 and 980 μeqv/l FFA respectively. Also used are two plasma samples each from pig, rat and man. Leading to a total of 12 plasma samples for use as quality controls. Furthermore, two independently manufactured solutions of the Wako calibrator (250 and 750 μeqv/l) are also used as quality control samples.

Analysis of the 12 plasma quality controls (two each from cattle, pig, rat and man) are repeated four times per day on four consecutive days. Analyses of the two commercial quality controls are repeated six times per day on four consecutive days. Intra and inter assay coefficient of variation are based on four days of analyses of the plasma (n = 48) and commercial (n = 48) quality controls.

2.2.4.3 Assay protocol

The assay was carried out using the ADVIA 1650 autoanalyser (Bayer, North Carolina, USA). Fifteen μL of sample, quality control or calibrator was mixed with 50 μL reagent

A and 50 µL reagent B. Plasma samples, quality controls and calibrators were loaded accordingly and the assay run.

2.2.4.4 Reliability criteria

A representative standard curve for FFA is given in *Figure 2.5*. This is the mean standard curve for the assay over the period of time that FFA was tested in these samples. The 14 quality control samples (See 2.3.4.2) are monitored and the ADVIA 1650 is recalibrated if these samples are outside of the accepted limits (± 2 standard deviation).

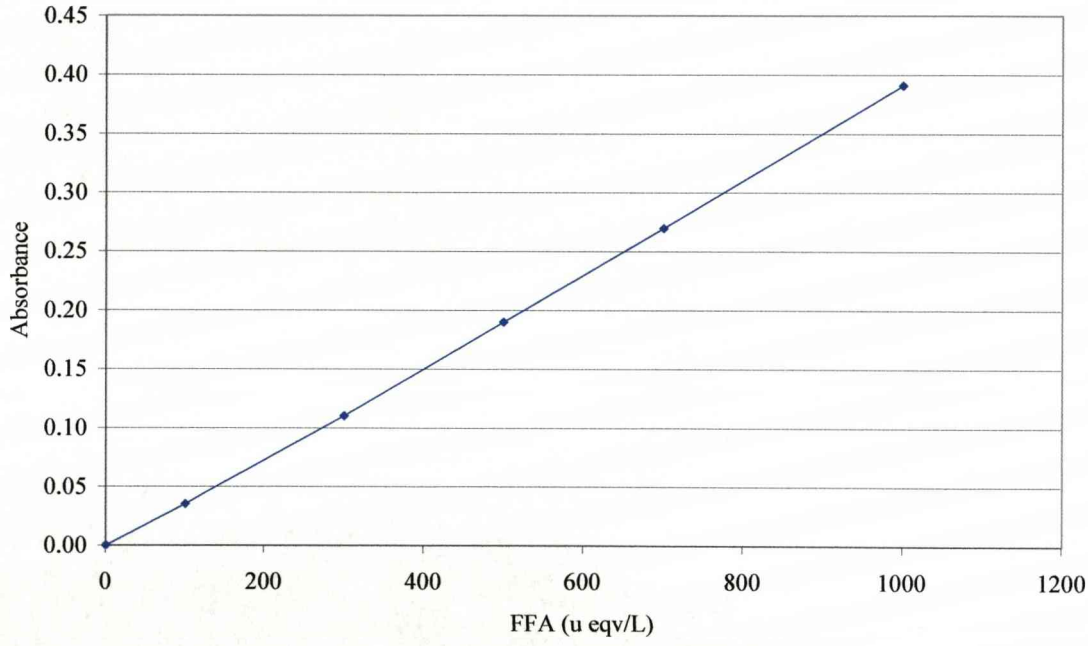

Figure 2.5 Representative standard curve for the FFA assay (obtained from the ADVIA 1650 autoanalyser output).

2.2.4.5 Sensitivity

The working range of the assay 0-1200 µeqv/L. See 2.1.5 for sensitivity definition. The sensitivity of the assay was approximately 5 µeqv/L (Larsen, T.; personal communication).

2.2.4.6 Precision

The intra-assay coefficients of variation for the low and high quality controls were 0.76 % and 0.90 % respectively (calculated using all assay data). The inter-assay coefficients of variation for the low and high quality controls were 3.09 % and 1.74 % respectively. All results above 1200 μeqv/L were re-assayed.

2.2.5 Glucose

Glucose content was determined using a commercial assay kit (Glucosehexokinase II; Bayer, North Carolina, USA) adapted to automated running on a Advia 1650 Bayer Opera system (Bayer, North Carolina, USA).

2.2.5.1 Reagents

Reagents were made up according to manufacturer instructions (*Table 2.11*).

Table 2.11 Reagents in the glucose assay kit (Bayer, North Carolina, USA).

Item	**Components**	**Concentration**
Reagent 1: 4 x 68mL	ATP (Adenosine triphosphate) NAD (Nicotinamide adenine dinucleotide) Sodium azide Buffer	4 mmol/L 3.21 mmol/L 0.05 %
Reagent 2: 4 x 19mL	ATP NAD Sodium axide Buffer	4 mmol/L 3.21 mmol/L 0.05 %
R2 mix:	Hexokinase (microbial source) G6P-DH (Glucose-6-phosphate dehydrogenase; microbial source) Sodium azide	≥25 U/mL ≥75 U/mL 0.09 %

Reagents were prepared, according to instructions, with the kit solutions (*Table 2.12*).

Table 2.12 Preparation of reagent 1 and reagent 2 (Glucosehexokinase II; Bayer, North Carolina, USA)

Reagent	**Instructions**
Reagent 1	Reagent 1 is ready to use after gentle mixing to ensure homogeneity and dislodge air bubbles. Store at 2-8 °C and use within 60 days.
Reagent 2	Empty one R2 mix vial into the R2 container and rinse the vial several times with R2 solution and emptying into the R2 container. Store at 2-8°C and use within 60 days.

2.2.5.2 Calibrators and quality control

A glucose standard solution was obtained from Bayer (Ref 09784096 Prod no T03-1291-62; Bayer, North Carolina, USA) for calibration and instruction followed. Plasma from cattle, pig, rat and man was used to prepare low and high glucose quality control samples. Leading to a total of 12 plasma samples for use as quality controls. Furthermore, two manufactured solutions Bayer Assayed Chemistry Control 1 and 2 (Ref 05788372 Prod no T03-1220-62; Ref 00944686 Prod no T03-1221-62) are also used as quality control samples.

Analysis of the 12 plasma quality controls (two each from cattle, pig, rat and man) are repeated four times per day on four consecutive days. Analyses of the two commercial quality controls are repeated six times per day on four consecutive days. Intra and inter assay coefficient of variation are based on four days of analyses of the plasma (n = 48) and commercial (n = 48) quality controls.

2.2.5.3 Assay protocol

The assay was carried out using the ADVIA 1650 autoanalyser (Bayer, North Carolina, USA). Samples, quality controls, calibrators and reagent 1 and 2 are loaded into the ADVIA 1650 and the assay set to run.

2.2.5.4 Reliability criteria

A representative standard curve for glucose is given in *Figure 2.6*. This is the mean standard curve for the assay over the period of time that glucose was tested in these samples. The 14 quality control samples are monitored and the ADVIA 1650 is recalibrated if these samples are outside of the accepted limits (± 2 SD).

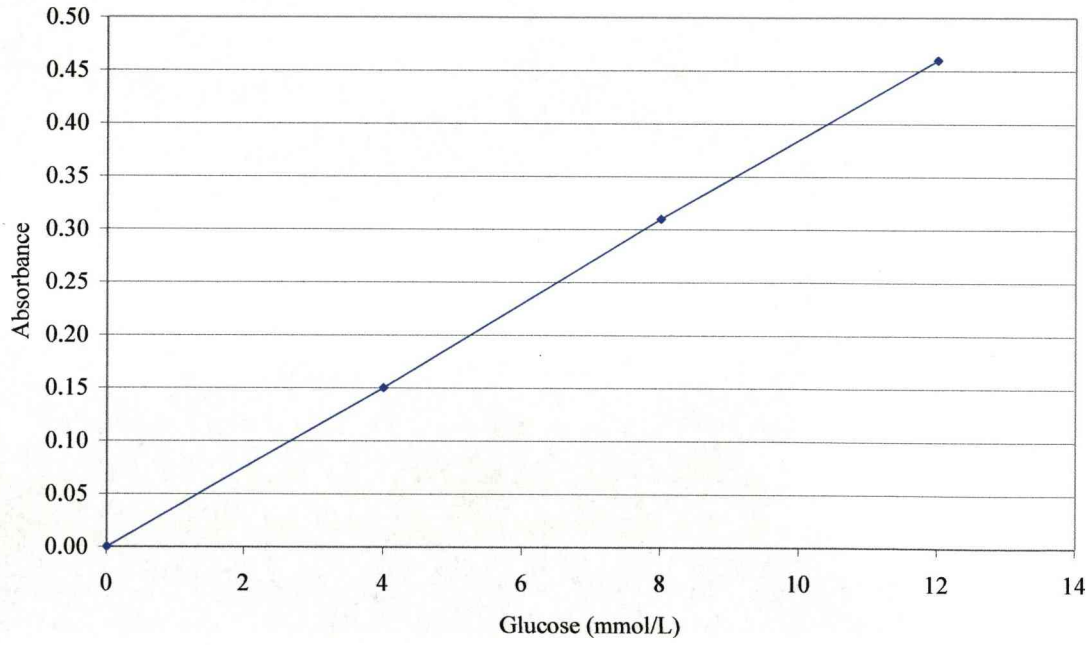

Figure 2.6 Representative standard curve for the glucose assay (obtained from the ADVIA 1650 autoanalyser output).

2.2.5.5 Sensitivity

The working range of the assay was 0-38.9 mmol/L. The sensitivity (See 2.1.5 for definition) of the assay was 0.1 mmol/L (Larsen, T., personal communication).

2.2.5.6 Precision

The intra-assay coefficients of variation for the low and high quality controls were 1.20 % and 1.30 % respectively (calculated using all assay data). The inter-assay coefficients of variation for the low and high quality controls were 1.94 % and 1.42 % respectively. All results above 38.9 mmol/L were re-assayed.

Chapter 3: DATABASES

In this chapter the databases used in this thesis will be described. The selection of farms and animals, farm management systems, data collection and milk and blood sampling protocols will be described.

3.1 INTRODUCTION

The use of databases to monitor health and fertility of dairy cattle in the UK and elsewhere, both phenotypically and genetically, have been widely described (e.g. Wiggans, 1994; Pryce *et al.*, 1998; Esslemont & Kossaibati, 2000; Royal *et al.*, 2000b; Royal *et al.*, 2002a; Royal *et al.*, 2002b; Petersson *et al.*, 2006a). Disease incidence, health and fertility parameters are routinely recorded by veterinarians and farmers in the Nordic countries, for example in Sweden milk recording, artificial insemination and disease recording data are combined in a central cattle data base managed by the Swedish Dairy Association (Swedish Dairy Association, Eskilstuna, Sweden), thus large databases are much more readily available and usable than in the UK (Heringstad *et al.*, 2000; Philipsson & Lindhe, 2003). Recording of disease and fertility traits is not compulsory in the UK although fertility and some diseases such as mastitis are monitored widely by e.g. NMR (National Milk Records, Chippenham) and Interherd (National Milk Records, Chippenham).

One advantage of such databases is that the use of field data, although perhaps collected in a less controlled environment, is more representative of the commercial environment and highlights, that for many traits, there is a genotype by environment interaction. A genotype by environment interaction describes the concept that animals differ in their ability to perform, phenotypically express their genotype, in different environments (reviewed by Bryant *et al.*, 2005) where environmental differences can refer to temperature, photoperiod, feeding level etc. Caution should be taken when using databases collected in a controlled experimental situation because genetic variation and correlations found may not be apparent in a commercial dairy herd where environmental

variation, due to for example management and feeding, is much greater. In contrast however data collected under strict experimental conditions often shows less environmental variation which allows genetic variation and relationships to be seen which otherwise may not be present in field data. Clearly there are advantages to both types of data, field and experimental, however when large datasets are needed the principal limiting factor is cost. Provided that care is taken when interpreting results then both types of study can be equally valid.

This thesis will describe analyses conducted on three databases (D1, D2 and D3). The first database (D1) represents animals monitored as part of a DEFRA supported project ("Prepubertal Selection for Daughter Fertility in Dairy Bulls" LS3204) between 2003-2006 from a controlled breeding programme (Cogent Breeding Ltd, Aldford, Cheshire, UK). This fortunately facilitated the construction of a database with a good genetic structure for genetic analysis (See section 3.2.1). The second dataset (D2) was also constructed using financial support from DEFRA between 1996 and 2000 (MDC/MAFF Contract LK 0605). The data was collected using the same experimental protocol as that described for D1 using 7 commercial UK dairy farms and thus combination of D1 and D2 was possible (See section 3.2.2). Finally, the third dataset (D3) represents data collected during a Danish project investigating physiological indicator traits in Danish dairy cattle breeding (1997-2002; Danish Institute of Agricultural Sciences; Løvendahl and Sørensen, 2001). The research was carried out at the Danish Institute of Agricultural Sciences (Danish Institute of Agricultural Sciences, Research Centre Foulum, Blichers Allé, Postbox 50, DK-8830, Tjele, Denmark) and the data structure has similar advantages to D1 (See section 3.2.1). Research relying on construction of databases of this nature is extremely costly, hence these costs have been overcome in part in the research described herein by using databases collected and financed from previous studies.

3.2 OVERVIEW OF DATASETS

3.2.1 Dataset 1

The analysis of this data is described in Chapters 5 and 6. The data was collected over a 4 year period (2002-2006; *Figure 3.1*).

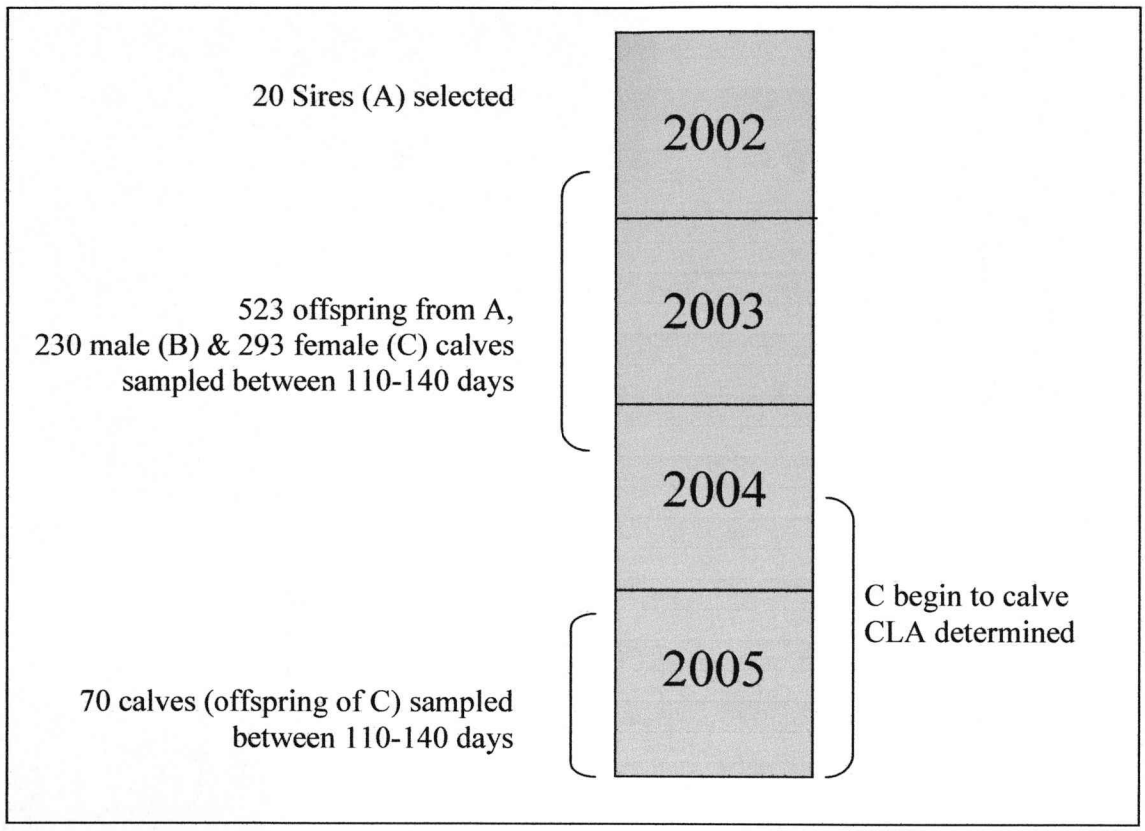

Figure 3.1 Overview of the sequence of data collection for Dataset 1.

3.2.1.1 Experimental design and selection of animals

All animals used in this study were provided by Cogent Breeding Ltd. and Grosvenor Farms Ltd. Cogent Breeding Ltd. is a dairy cow breeding company established in 1995 with the aim of providing UK dairy farmers with top quality UK proven sires. The company has a purpose built bull stud, the first commercial sexed semen service and, ran the world's largest nucleus programme and evaluation herd between 1995 and 2005.

Many of the animals selected as calves for the study had ownership transferred to Grosvenor Farms Ltd. (four dairy units) as milking heifers.

This study utilises blood samples collected during a concurrent study (Royal *et al.* unpublished results) investigating the LH response to GnRH challenge, therefore the number of animals used were chosen to meet the requirements of the original study. However the optimal design theory, used to determine the number of animals needed and the family structure, will be briefly described. This states that when using half sib correlation to estimate heritability, it is optimal that $nt = 1$ where n is the number of offspring and t is the half sib correlation coefficient ($¼h^2$; Robertson, 1959). The heritabilities to be estimated were assumed to be in the range 0.2-0.5 (based on the fact that other studies had found the heritability of LH and testosterone response to GnRH in cattle and sheep were in the same range; Haley *et al.*, 1989; Haley *et al.*, 1990; Mackinnon *et al.*, 1991). According to the optimal design theory, 8-20 offspring per sire for 15-20 sires were required creating a total of 160-400 offspring. Sires were chosen that had been widely used in the breeding program and that had the correct number of offspring. In total 20 sires were chosen with a total of 230 male and 293 female calves. The calves were distributed between the 20 sires as illustrated in *Figure 3.2*. Ancestry of these animals was traced back at least three generations to construct a pedigree file.

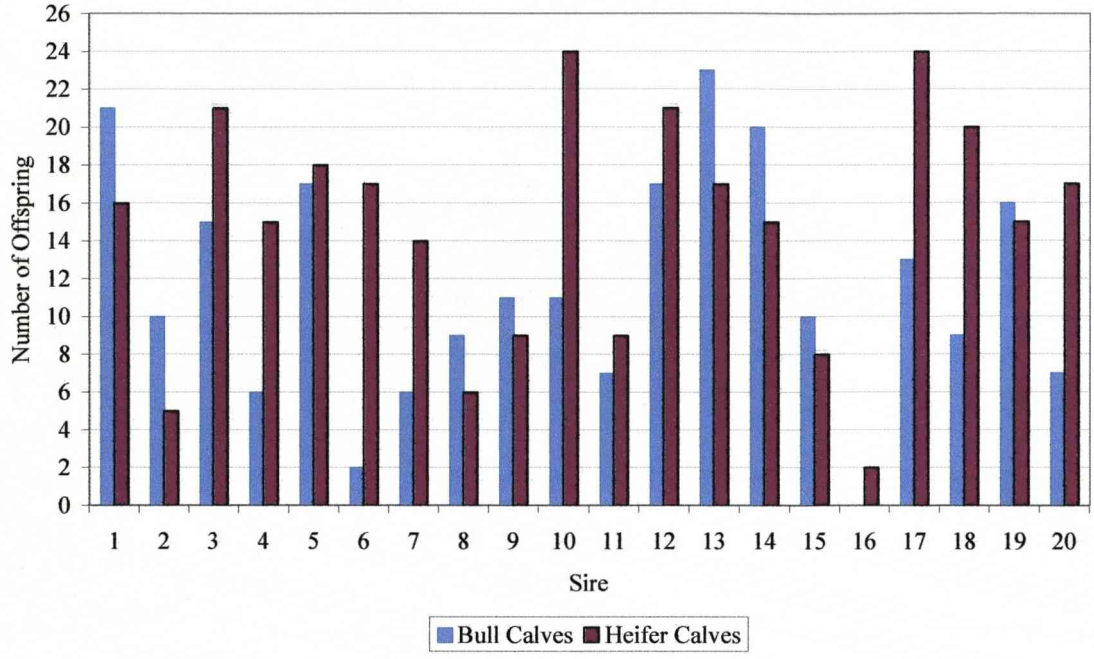

Figure 3.2 The distribution of 523 calves selected between the 20 sires used in phase 1.

The second phase of the study involved monitoring offspring of the 293 female calves from phase 1 of the study. A further 70 (43 female; 27 male) calves were monitored during phase 2 of the study resulting in a total of 593 animals in Dataset 1. *Figure 3.3* illustrates the animal distribution across the two phases of the study.

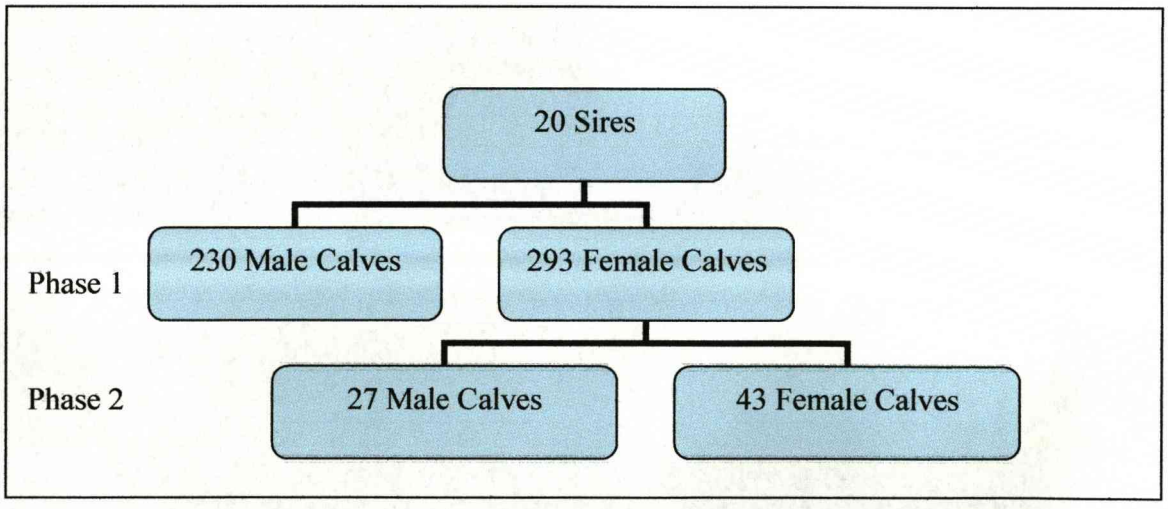

Figure 3.3 Illustration of first and second phase animal distribution.

3.2.1.2 Animal management

Calves were fed colostrum and milk replacer (Volac heiferlac instant, Volac international, Orwell, UK). In addition, barley straw was fed *ad libitum* and calf pellets (HST feeds, Crewe, UK) were fed in increasing amounts according to age and weight until weaning was complete between 9-12 weeks of age. Post weaning calf pellets, hay, straw, grass and maize silage were fed in varying quantities according to age and weight.

Heifers were reared at rearing units where diet varied according to different management practices, however the diets were based on concentrates, grass and maize silage and grazing during the summer. After calving heifers graduated onto one of four dairy units where they were fed a TMR (total mixed ration) containing grass silage, maize silage, brewer's grains, wheat/barley, rape, soya, bread, limestone/yeast and mineral supplements and were housed all year around with zero grazing.

Herd health was monitored and treated by weekly veterinary visits in addition to farm staff observation and treatment where appropriate. Reproductive problems were not treated for 40-50 days postpartum unless this compromised the animals' welfare and cows were left open for a minimum of 50 days. Heat observation was visual, by pedometer monitoring and by use of Kamar Heatmount detectors (Kamar Inc., Steamboat Springs, CO 80477, USA). Artificial insemination was carried out by trained farm staff at each of the units. There was no specific calving pattern at any of the four farms. Cows calved throughout the year with each farm tending to have slightly higher number of calvings over the winter months (Nov-Feb; *Table 3.2*).

3.2.1.3 Blood sampling

3.2.1.3.1 Protocol

As previously mentioned, this study utilises blood samples collected for a previous study however only the samples collected before the hormone challenge are used in the current

study. Calves were sampled between 110-140 days of age (average 123 ± 15 days). Phase 1 sampling was carried out at approximately one month intervals over a 16 month period until sufficient numbers of calves from each sire had been monitored in order to achieve the experimental design.

Male and female calves were housed at different units. On the sampling days the male calves were sampled in the morning and the females in the afternoon. Calves to be sampled were taken out of their normal pens and put in individual calf pens to be sampled. The protocol stated that calves were left to become accustomed to their surroundings for between 30-60 mins however on some occasions this did not occur. Animals were haltered and loosely tied except for when a blood sample was taken during which they were restrained. Blood samples were taken from the jugular vein at set time intervals by venepuncture into heparinized tubes (*Figure 3.4*).

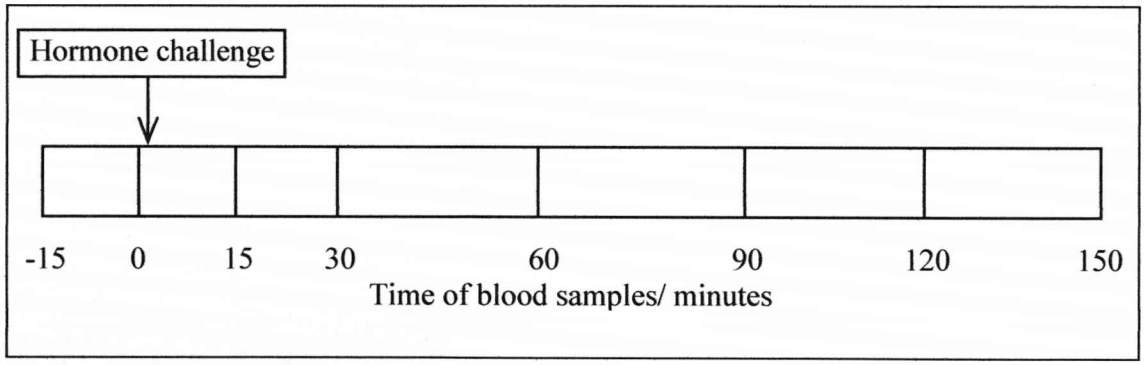

Figure 3.4 Timing of blood samples in relation to hormone challenge.

Immediately after the second blood sample (0 minutes) 1 ml of Fertagyl (equivalent to 0.1 mg GnRH; Intervet UK Ltd., Science Park, Milton Road, Cambridge) was given intramuscularly into the rump. Blood samples were centrifuged (3000 rpm, 15 min) and the plasma removed and frozen (-20 °C) until analysis. Only the first two blood samples, taken at -15 and 0 minutes relative to hormone challenge, were used in the present study (*Table 3.1*).

Table 3.1 Sampling day schedule, highlighting the samples used for growth hormone (GH), glucose, insulin, free fatty acids (FFA) and insulin like growth factor 1 (IGF-I) measurement in Dataset 1

Test	**Tube Code**	**Time relative to challenge (min)**	**Sample used for**
Baseline	1, 2	-15, 0	GH, glucose, insulin, FFA (both samples) and IGF-I (0 sample only)
Hormone challenge	3, 4, 5, 6, 7, 8	15, 30, 60, 90, 120, 150	Not used

3.2.1.3.2 Data collected

The unique eartag number, sex, date of birth, date of blood sampling, weight (using a weigh tape), dam, sire and maternal grandsire were recorded for all animals.

3.2.1.3.3 Practical problems

In April 2005 the breeding programme at Cogent Breeding Ltd. was terminated and links between Cogent Breeding Ltd. and Grosvenor Farms Ltd. were severed. This made blood sampling after this time difficult because calves born were not recorded at a central unit, many calves were sold and thus unavailable for sampling. This led to a reduced number of calves sampled in the second phase than expected and sampling ended prematurely in August 2005. However, this did not have an adverse effect on the research described herein.

3.2.1.4 Milk progesterone database

3.2.1.4.1 Sampling protocol

A number of the originally blood sampled female calves (See *Figure 3.1*) went on to be milk sampled at one of four dairy units. Thrice weekly representative milk samples (30

ml) were taken in plastic milk pots (Massmould, Luton, UK) each with one Lactab mark 3 (Thompson & Capper Ltd., Runcorn, UK), a milk preservative composed of 30mg potassium dichromate and 20mg salt. Samples were assayed at Nottingham University for progesterone using an enzyme linked immunosorbent assay (ELISA; See Chapter 2 for detailed assay method). Commencement of luteal activity postpartum (CLA) was then estimated (See Chapter 1.6.2). This was characterised as the number of days postpartum when the first of ≥ two consecutive milk samples measured ≥ 3 ng/ml of progesterone during three times per week sampling (Royal *et al.*, 2000b; *Figure 3.5*).

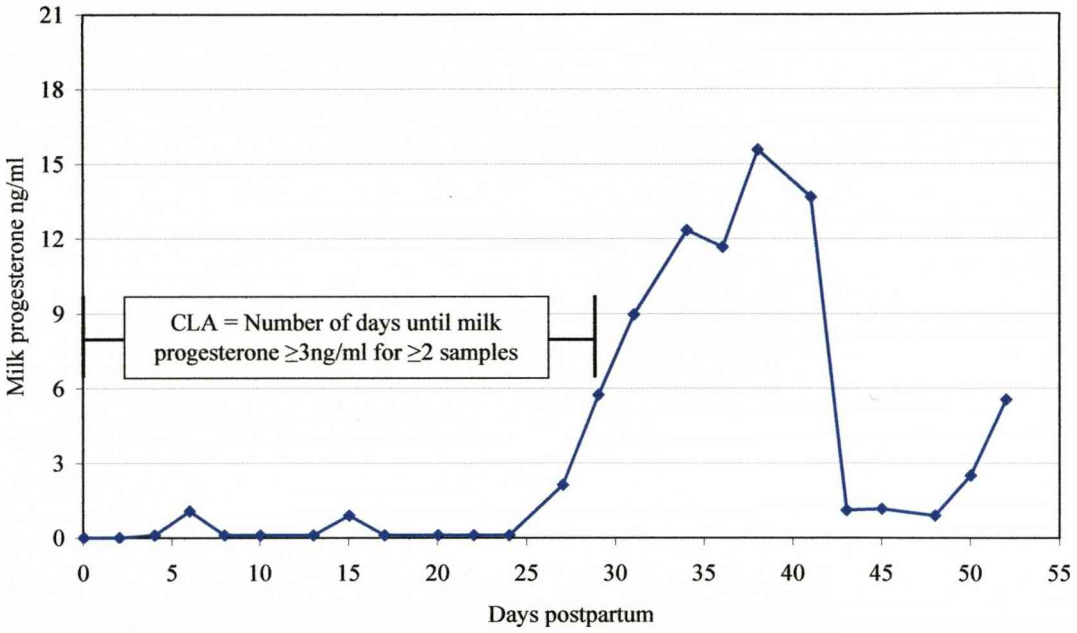

Figure 3.5 Example milk progesterone profile showing the interval to commencement of luteal activity postpartum (CLA) which in this example is 29 days.

Furthermore, a fertility measure introduced by Petersson *et al.* (2006a; 2006b; See Chapter 1.6.2) was calculated. This was determined as the percentage of milk samples with luteal activity (milk progesterone ≥ 3ng/ml) of all or a subset of the samples taken within 60 days post partum (PLA; *Figure 3.6*).

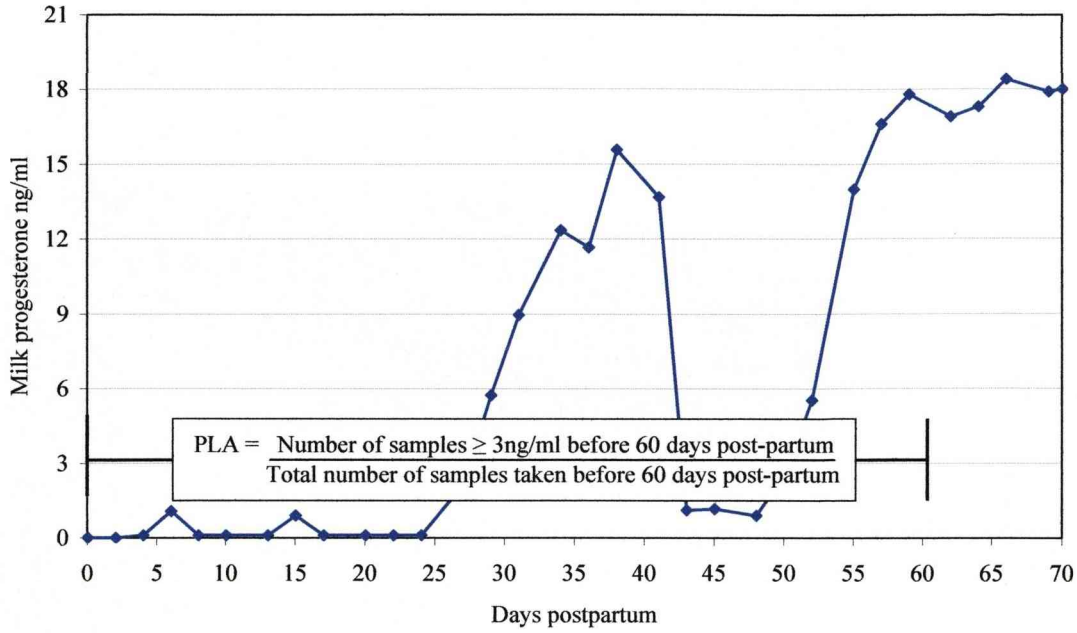

Figure 3.6 Example milk progesterone profile showing the percentage of milk samples with luteal activity up to 60 days postpartum (PLA), here PLA equals 37%.

PLA can be measured using a number of different milk sampling protocols. The first PLA measure (PLA_a) uses all milk samples collected within 60 days postpartum. In addition to this, PLA was calculated using the first milk progesterone sample per week (PLA_w), the first milk progesterone sample per fortnight (PLA_f) and a randomly selected sample during the first four weeks and thereafter taken at monthly intervals (PLA_m) representing once a week, twice a month and once a month sampling protocols.

The distribution of heifers that were milk sampled after calving, of the original 293 female calves blood sampled, on the four dairy farms, and the average CLA and PLA_a are shown in *Table 3.2*. From *Table 3.2* it is apparent that there is little difference between the average CLA ($P>0.05$) and PLA_a ($P>0.05$) for each farm.

Table 3.2 The number of heifers (of the original 293 female calves) at each of the four farms, plus the distribution (Dec, Jan & Feb = Season 1, Mar, Apr & May = Season 2, Jun, Jul & Aug = Season 3 and Sept, Oct & Nov = Season 4), average number of days postpartum until commencement of luteal activity (CLA; ± standard error) and average percentage (%) of samples with luteal activity upto 60 days postpartum using all milk samples (PLA_a; ± standard error)

Farm	**Number**	**Calving season**				**Average CLA (± s.e.)**	**Average PLA_a (± s.e.)**
		1	**2**	**3**	**4**		
A	95	19	18	27	27	40.12 ± 2.52	36.88 ± 2.28
B	55	18	13	11	13	34.38 ± 2.75	43.18 ± 3.49
C	43	11	8	9	13	43.16 ± 4.94	36.23 ± 3.83
D	34	12	5	7	10	33.37 ± 6.09	42.34 ± 4.05

3.2.1.4.2 Data collected

At parturition, the calving score (Berger, 1994; See section 1.4.2.3 for detailed description) in addition to reproductive health problems (e.g. retained fetal membranes and uterine infection) were recorded; general health was also recorded throughout the milk sampling period (eg. mastitis and veterinary treatments). Furthermore, reproductive information such as oestrus and artificial insemination (AI) were recorded (See *Table 3.3*). There was no significant difference ($P>0.05$) between the four farms for either the average interval to first oestrus or the average interval to first service.

Table 3.3 The number of animals (sampled in D1) at each farm, the average interval to first observed oestrus (days ± standard error) and interval to first service (days ± standard error)

Farm	Interval to first oestrus		Interval to first service	
	N	Average (± s.e.)	N	Average (± s.e.)
A	28	52.21 ± 7.36	88	66.20 ± 2.71
B	5	48.20 ± 7.64	49	66.29 ± 3.05
C	1	22.00 ± 0.00	35	73.54 ± 6.47
D	13	67.62 ± 3.88	29	65.41 ± 2.44
Total	47	55.40 ± 4.71	201	67.37 ± 1.83

3.2.1.4.3 Practical problems

Not all of the phase 1 heifers (72 out of 293) remained in the herd until their first lactation therefore it was not possible to assess their fertility.

3.2.1.5 Embryo flush and transfer database

At Cogent Ltd. multiple ovulatory embryo transfer (MOET) was carried out on selected females at 12 months of age. Of the 293 female calves that were sampled in phase one 102 were used as embryo donors. These animals were selected due to the superior genetic merit of their sire and dam. 153 of the 293 females were used as recipients of embryo's from donor cows. These recipients were chosen due to them having lower genetic potential and their age making them suitable to receive an embryo. Detailed information was collected on these animals regarding their response to superovulation and their success as recipients, however, this data is not analysed for this thesis.

3.2.1.6 Male fertility database

Of the 230 male calves that were sampled in phase one 42 entered the bull stud at Cogent Ltd. for semen collection at approximately 12 months of age. The remaining

male calves were sold or culled. Detailed information was collected on these animals regarding their semen parameters however in this thesis this data will not be analysed.

3.2.2 Dataset 2

The analysis of this data is described in Chapters 5 and 6. The study was conducted between 1996 and 2000 at the University of Nottingham (Royal, 1999).

3.2.2.1 Selection of animals

All animals were Holstein-Friesian heifer calves (n = 524) selected from seven commercial dairy units (*Figure 3.7*). The farms were chosen due to their high standard of record keeping and their willingness to be involved in the study. Calves were selected according to their age on day of sampling (120-140 days) and sire. The aim was to sample 8-20 offspring per sire family (See section 3.2.1.1). As in D1 the ancestry of these animals was traced back at least three generations to construct a pedigree.

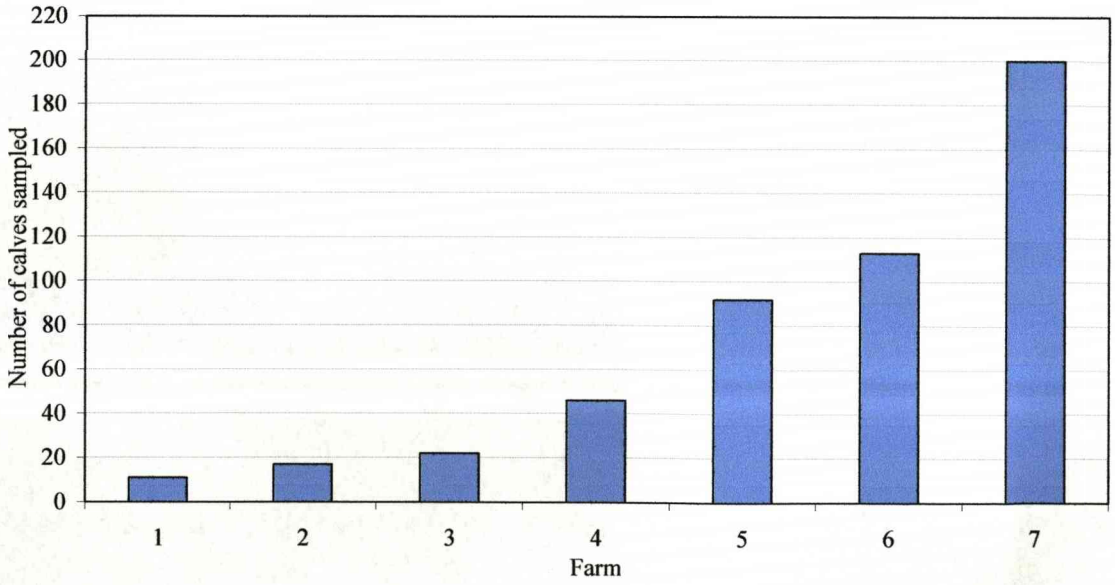

Figure 3.7 Number of female calves sampled at each farm in Dataset 2.

3.2.2.2 Animal management

Calves were raised at the individual farms as replacement heifers. All animals were reared on milk powder and had been weaned by the time of blood sampling. The diet varied depending on time of year and the different management practices between individual farms, however the majority were fed dry cake rations and hay according to approximate weight and age. The calves that remained at the individual farms as heifers were milk sampled in their first lactation.

3.2.2.3 Sampling protocol

Sampling protocol is similar to that in D1 (See section 3.2.1.3) except that the first blood sample was taken at -30 minutes and no sample is taken at +15 minutes (See *Figure 3.8*). As in Dataset 1 not all of the blood samples collected were used in the present study (*Table 3.4*).

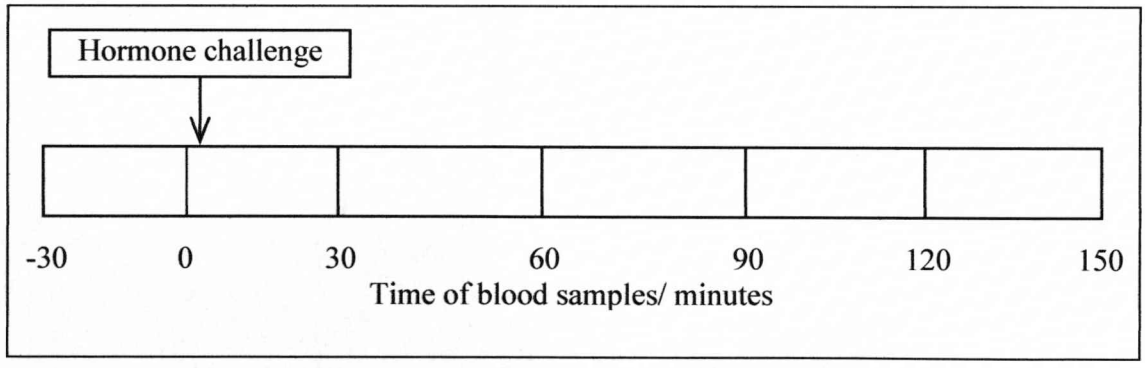

Figure 3.8 Timing of blood samples in relation to hormone challenge

Table 3.4 Sampling day schedule, highlighting the samples used for growth hormone (GH), glucose, insulin, free fatty acids (FFA) and insulin like growth factor 1 (IGF-I) measurement in Dataset 2.

Test	**Tube Code**	**Time relative to challenge (min)**	**Sample used for**
Baseline	1, 2	-30, 0	GH, glucose, insulin, FFA (both samples) and IGF-I (0 sample only)
Hormone challenge	3, 4, 5, 6, 7	30, 60, 90, 120, 150	Not used

3.2.2.4 Milk progesterone database

Milk samples were collected from the calves blood sampled if they remained in the herd as heifers. On only four (farms 4, 5, 6 and 7; *Figure 3.7*) of the seven farms, originally blood sampled, was milk sampling carried out. The milk collection protocol was identical to that described for D1 (See 3.2.1.4). The distribution of heifers that were milk sampled after calving, of the original 524 female calves blood sampled, on the four dairy farms, the season of calving, the average CLA and PLA_a are shown in *Table 3.5*. From *Table 3.5* it can be seen that there was little difference between the average PLA_a of each farm however there was significant difference between the average CLA ($P<0.025$) of the four farms. Farm A has a higher average CLA and standard error, compared to B, C and D, however this is likely due to the small number of animals (n = 4).

Table 3.5 The number of heifers out of the original 524 female calves at each of the four farms, plus the distribution (Dec, Jan & Feb = Season 1, Mar, Apr & May = Season 2, Jun, Jul & Aug = Season 3 and Sept, Oct & Nov = Season 4), average number of days postpartum until commencement of luteal activity (CLA; ± standard error) and average percentage of samples with luteal activity upto 60 days postpartum using all milk samples (PLA_a; ± standard error)

Farm	**Number**	**Calving season**				**Average CLA (± s.e.)**	**Average PLA_a (± s.e.)**
		1	**2**	**3**	**4**		
A	4	3	1	0	0	47.50 ± 17.96	---
B	39	1	2	12	20	20.85 ± 1.42	59.55 ± 3.34
C	76	25	16	28	3	30.34 ± 1.73	46.59 ± 2.64
D	114	23	31	15	45	26.40 ± 1.50	54.02 ± 4.35

3.2.2.5 Data collected

As for D1 (See section 3.2.1; See *Table 3.6*). The difference between average interval to first oestrus for each farm was not significant whereas the difference in average interval to first service for each farm was significant ($P<0.005$). Farm B had a much shorter average interval to first service than farms A, C and D.

Table 3.6 The number of animals sampled in D2 at each farm, the average interval to first observed oestrus (days ± standard error) and interval to first service (days ± standard error)

Farm	**Interval to first oestrus**		**Interval to first service**	
	N	**Average (± s.e.)**	**N**	**Average (± s.e.)**
A	2	35.00 ± 0.00	2	79.00 ± 2.00
B	9	35.11 ± 2.46	37	58.86 ± 2.17
C	57	40.93 ± 2.34	71	78.58 ± 1.69
D	0	---	40	79.53 ± 7.31
Total	68	39.99 ± 2.00	150	73.97 ± 2.27

3.2.3 Dataset 3

The analysis of this dataset is presented in Chapter 4. The data was collected during a study investigating physiological indicator traits in Danish cattle breeding (1997-2002; Danish Institute of Agricultural Sciences; Løvendahl and Sørensen, 2001). All procedures were approved by the Danish Animal Experiments Inspectorate and complied with the Danish Ministry of Justice (Law 382, 1987; Act 739, 1988; Act 333, 1990) concerning animal experimentation and care of experimental animals.

3.2.3.1 Selection of animals and experimental design

A cohort quantitative genetic study was designed. All animals were (n = 1498 male calves) from a Danish progeny testing scheme. Calves were Danish Holstein (n = 1047), Danish Jersey (n = 200) and Red Dane (n = 251). Physiological data, including plasma samples, was collected on the males as calves (269.5 days of age ± 11.1; *Table 3.7*) and furthermore, progeny test results for these animals were collated at a later stage (Danish Cattle Federation, Aarhus N, Denmark). Ancestry was traced back at least three generations to construct a pedigree file with 59243 animals.

Table 3.7 Number and age (± standard deviation) of male calves of each breed sampled at each farm.

Farm	**Breed**	**Number**	**Mean age /days ± s.d.**
A	Red Dane	23	266.65 ± 7.17
	Danish Holstein	193	264.00 ± 8.76
	Danish Jersey	16	264.94 ± 4.58
B	Red Dane	228	269.81 ± 10.86
	Danish Holstein	508	269.24 ± 9.93
	Danish Jersey	184	271.71 ± 11.99
C	Danish Holstein	269	273.12 ± 13.27
D	Danish Holstein	77	267.84 ± 10.18
Total		1498	269.49 ± 11.13

3.2.3.2 Animal management

Calves were housed at 4 experimental stations (A, B, C & D) where they arrived ideally before 3 months of age but calves arriving up to 5 months of age were still accepted for further testing. Calves were born in several private herds and transferred to the stations following the necessary health checks. A diet based on dried grass pellets supplemented with barley straw was fed *ad libitum* and water was freely available.

3.2.3.3 Experimental procedure

Two blood sampling protocols were used during this study; in protocol 1 blood samples were taken by jugular venepuncture (1997-1999) and in protocol 2 by jugular cannulation (1999-2002). Eight days before the day of sampling (day 0) calves were grouped into batches (5-24 calves) and weighed (*Figure 3.9*). Normal feeding occurred on days -8, -7, -6 and -5. On days -4, -3, -2 and -1 calves were fed to cover maintenance only in a single meal at 15.30 h in order to avoid interference between expected feeding time and circulating hormone concentration and so that time elapsed since last meal (16 ½ hours) was the same in all animals on the day of sampling. On day -1 calves were cannulated and on day 0 sampling was carried out and normal feeding resumed at 15:30h.

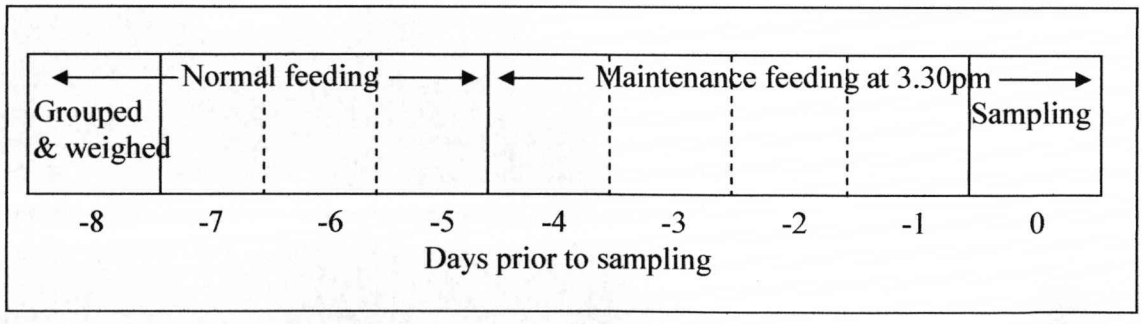

Figure 3.9 Feeding protocol relative to blood sampling on day 0

Various blood samples were taken during the day including pre and post growth hormone releasing hormone (GHRH), adrenalin, and glucose challenge. *Table 3.8*

indicates which samples were used for the current analyses. Cannulae were filled with isotonic saline supplemented with heparin between sampling. Blood was chilled on ice and plasma separated by centrifugation (2000×g, 4°C, 20 min) and stored frozen (-20 °C) until assayed (see Chapter 2 for assay details).

Table 3.8 Sampling schedule highlighting samples used for growth hormone (GH), glucose, insulin and FFA measurement in protocol 1 and 2.

Protocol 1 1997-1999		
Test	**Time**	**Sample used for**
Baseline	08.00	Growth hormone
GHRH challenge	08.15, 09.00	Not used
Baseline	11.00	Not used
Adrenalin challenge	11.15, 11.30	Not used
Baseline	13.30	FFA, glucose and insulin
Glucose challenge	13.45, 14.30	Not used
Protocol 2 1999-2002		
Baseline	07.45, 08.00	Growth hormone
GHRH challenge	08.10, 08.20, 08.40, 09.00	Not used
Baseline	10.45, 11.00	Not used
Adrenalin challenge	11.10, 11.20	Not used
Baseline	13.15	FFA, glucose and insulin
Glucose challenge	13.40, 13.50, 14.10, 14.30	Not used

3.2.3.4 Female fertility breeding values

Estimated breeding values (EBV) for female fertility (FertEBV) were available for 810 (mean ± s.d., 96.16 ± 9.54) of the 1498 originally sampled male calves (estimated from approximately 100 daughters each; calculated by the Danish Cattle Federation, Aarhus N, Denmark). At the time of writing this thesis the fertility index linked information on three fertility measures routinely recorded in Denmark which are: days from first to last insemination in heifers and cows, and days from calving to first insemination in cows

(See Chapter 1). The three breeding values are weighted according to economic values and combined to give the FertEBV. The FertEBV is standardized to give a mean of 100, a standard deviation of 5 and higher values are indicative of better fertility.

3.3 DISCUSSION

Datasets 2 and 3 had been collected previously in different studies therefore practical problems with collection were in the past and therefore could not be dealt with. A disadvantage to the use of such data (D2 and D3) is that the purpose of its collection is specific to another study so analyses are limited by the structure and content of the already complete dataset. However the use of D2 and D3 has been very beneficial in this research. Fortunately D2 and D3 were both genetically structured databases collected for genetic analyses. Both D2 and D3 contained data from dairy breeds and from pre-pubertal animals. As D2 was collected before D1 but followed the same protocol the two relatively small datasets (D1 n = 593 & D2 n = 524) could be combined (See Chapter 5) to give a larger dataset. The use of D3 (n = 1498) has allowed the comparison of Danish and UK dairy cows. However, in such comparison and with the use of several datasets to increase the number of animals (often a limiting factor in genetic analyses) it is important to be aware of differences such as management, breeds, country, climate, selection pressures etc as these can affect results.

The three datasets (D1, D2 and D3) contain similar information, however, the breeds used are different. The largest difference is in D3 in which, in addition to Danish Holsteins (n = 1047), Danish Jersey (n = 200) and Red Danes (n = 251) were sampled. The majority of animals in D3 were Danish Holstein and this breed is comparable to the UK Holstein for production (average milk yield UK Holstein 8212 kg/year in 2005; Danish Holstein 9122 kg/year in 2005; NMR, Fox Talbot house, Greenway Business Park, Belinger Close, Chippenham, UK; Danish Cattle Federation, Aarhus N, Denmark, respectively). The average milk yield in the Red Dane (8380 kg/year in 2005; Danish Cattle Federation, Aarhus N, Denmark) is similar to the UK Holstein and the Danish Jersey is lower (6346 kg/year in 2005; Danish Cattle Federation, Aarhus N, Denmark).

Although the differences in milk production between breeds in D1, D2 and D3 are small there could be differences in fertility. The average conception to first service in the UK dairy population is low at approximately 40% (37%, n = 2471, Northern Ireland; Mayne *et al.*, 2002; 39.7%, n = 714, 1995-1998; Royal *et al.*, 2000a). Dairy cattle fertility is similar in Denmark with average days to first service of 86 and 98 for the Danish Jersey and Danish Holstein respectively in 2005 (Danish Cattle Federation, Arhus N, Denmark; average days to first service 78 in UK 1995-1998, Royal *et al.*, 2000a). Denmark has been more aware of fertility management than the UK in that they have selected for fertility for some time and they place almost equal emphasis on production, durability and health and reproduction in their commonly used "S index" (Miglior *et al.*, 2005). Whilst animals in D1 and D2 have similar CLA and PLA following first calving, the actual fertility data is not available for the female offspring of calves in D3 and it is not possible to compare the fertility index data. It would appear that despite small differences between the Danish and UK dairy industries (See Chapter 1.2) they are similar enough to allow the use and comparison of D3 with UK datasets D1 and D2.

D1 and D2 both sample Holstein-Friesian calves, however, over different time periods (D1 2002-2006; MOET breeding scheme, D2 1996-2001; 7 commercial dairy herds). It is possible that there may be a slight breed difference between these two datasets due to the increasing use of Holstein sires and the reduction of British Friesian genes in the UK black and white dairy population. Over the last 30 years there has been a large increase in the percentage of North American Holstein genes in the UK herd. A study by Roughsedge *et al.* (1999) found that in 1997 the UK Holstein-Friesian breed was on average 24% British Friesian and 76% North American Holstein compared to 96% British Friesian in 1967. It is likely that the proportion of North American Holstein genes has further increased in the 10 years since this review. Although the introduction of North American Holstein genes into the UK herd has been central to the increases in milk production unfortunately it has also, in part, caused the low fertility seen at present (average conception rate to first service 37.1 % n = 2471; Mayne *et al.*, 2002). The average percentage Holstein genes of the sires in D1 and D2 were available and comparison shows that the difference between the two datasets was negligible (average

sire percentage Holstein ± standard error, D1 99.41 ± 0.08; D2 99.88 ± 0.04) and unlikely to affect the data.

Although neither animals in D1 nor D2 followed a specific calving pattern there tended to be slightly more calvings during seasons 1 (December, January and February) and 4 (September, October and November; *Table 3.2 & 3.5*). Animals in D1 were housed all year round therefore postpartum diet would be the same whenever calving occurred. However, animals in D2 were grazed from late spring to early autumn (dependent on management and weather) between morning and afternoon milking thus postpartum diet would vary dependent on time of calving. Winter and spring calving animals that are turned out to graze in spring sometimes experience lower milk yield due to reduced energy intake compared to housed animals (Chapa *et al.*, 2001). Concentrate supplementation is carried out to improve milk yield in grazed animals however this can be problematic. High crude protein and low energy diets can cause fertility problems such as lower conception rate to first AI and increased days open (McCormick *et al.*, 1999). Furthermore, grazing with concentrate supplementation can lead to high plasma urea nitrogen and milk urea in comparison to housed animals (McCormick *et al.*, 1999). In contrast to these findings grazing can be beneficial to young heifers. In comparison to housed heifers those grazed during rearing, before and after first calving have lighter calves and higher dry matter intake both pre and postpartum due to a better developed rumen and lower condition score at first calving (Troccon, 1993).

In addition to CLA and PLA, traditional fertility parameters were recorded for some, but unfortunately not all, of the animals in D1 and D2 following first calving (*Table 3.3 & 3.6*). The interval to first observed oestrus after first calving was longer in D1 than D2 (n = 47, 55.40 ± 4.71 days; n = 68, 39.99 ± 2.00 days respectively, $P<0.025$; *Table 3.3 & 3.6*) whilst the interval to first service after calving was longer in D2 than D1 (n = 201, 73.97 ± 2.27 days; n = 150, 67.39 ± 1.83 days respectively, non significant). Furthermore, the interval to first oestrus and first service in both D1 and D2 are comparable to other studies (Shipka & Ellis, 1999; Royal *et al.*, 2000b). In the commercial breeding company herds (D1) oestrus did not occur or was not detected until

approximately 55 days postpartum. This may be due to lower fertility in these animals or perhaps heat detection is not the main priority in these herds (D1). Whereas, perhaps in smaller independent farms (D2) heat detection is better. Despite the longer interval to first oestrus after calving in D1 these cows were served on average 6 days sooner than cows in D2. The animals for which interval to first service data is available were not treated for reproductive disorders consequently, first service was carried out after a natural oestrus. Therefore regardless of differences in the days till first observed oestrus postpartum (D1 and D2), the oestrus that preceded AI occurred approximately the same number of days postpartum.

Also to be considered in the use of several datasets (D1, D2 and D3) is that experimental protocol differences may produce differences in the extent of "stress" response in the animals. Stress can affect concentrations of many hormones and metabolites including cortisol, glucose and FFA (reviewed by Obernier & Baldwin, 2006; See Chapter 5.4.3 for detailed discussion of stress affects). Calves in D3 were sampled for a longer period of time than animals in D1 and D2 therefore allowing them longer time to become adjusted to the sampling procedure and environment. However, calves in D3 had been fasted overnight prior to sampling therefore were under nutritional stress which may have affected hormone concentrations. In D1 and D2, the experimental protocol required that calves be left for 30-60 minutes prior to blood sampling to become accustomed to the experimental site however in D1 in some cases this was not the case. This was partly due to animals in D1 being from a commercial breeding company therefore we had less opportunity to manage the protocol prior to sampling. It is likely, in these cases, that these calves were more stressed than their counterparts for which experimental protocol was followed more stringently (See Chapter 5.4.3). Furthermore, the method of blood sampling affects hormone concentration results. More accurate sampling results are achieved by cannulation (Løvendahl & Sørenson, 2001) and perhaps sampling method also affects stress levels. The samples in D1 and D2, and the majority of samples in D3 were collected by the same method, of jugular venepuncture, whilst the latter half of samples taken in D3 (1999 – 2002) were by jugular cannulation which should be considered in the data analyses.

3.4 FUTURE WORK

If these datasets were constructed again it would be useful to collect additional data such as milk production information for females in D1 and D2 to allow the direct comparison of plasma hormone and metabolite concentrations with actual milk yield. The period of time that calves were left to become accustomed to their surroundings before blood sampling was carried out in D1 and D2 should have been recorded to allow the affect of stress to be assessed and accounted for in the analyses. Also, ideally more calves would have been sampled, particularly males in D1. This was not possible at the time and of course cost and time are limiting factors in all such studies.

Further data is available in D1 which could be analysed in the future. Several of the female calves sampled in D1 were used as embryo donors and as embryos recipients. Therefore analyses could be carried out on the response to super-ovulation and the success as a recipient. Also, of the 230 male calves that were sampled in D1, 42 entered the bull stud at Cogent Breeding Ltd. for initial semen collection at approximately 12 months of age. Further information is available on semen quality and collection data on all bulls at the stud. Genetic variation in semen parameters could be investigated and also whether there is a link to hormone and metabolite concentrations at 4 months of age. The LH response to a GnRH challenge has been measured in all animals in D1 and D2. The link between this and concentrations of FFA, glucose, GH, IGF-1 and insulin could be investigated in the future. Furthermore, production data could be collated for the females in D1 and D2, and the presence of genetic and phenotypic relationship with FFA, glucose, GH, IGF-1 and insulin could be examined.

This chapter illustrates that the datasets used in this thesis are varied in size, breed and data collected. Although there can be difficulties when collecting and using large quantities of data, the use of such datasets has allowed different analyses to be carried out and data to be compared.

Chapter 4: METABOLIC REGULATION IN DANISH BULL CALVES AND ITS RELATIONSHIP TO THE FERTILITY OF THEIR OFFSPRING

4.1 INTRODUCTION

Most dairy systems aim to achieve a 365 day calving interval. This requires the animal to conceive by 80 days postpartum and yet during this time the cow may be producing up to 45 litres of milk per day. In the past, most dairy cattle breeding programs have been focused on increasing production. In fact milk yield over the last 30 years has increased dramatically particularly in the Holstein-Friesian population. Although this is obviously still a major economic factor today, there is growing evidence of unfavourable genetic correlations between production traits and others of economic importance such as fertility (e.g. Hoekstra *et al.*, 1994; Pryce *et al.*, 1997; Veerkamp *et al.*, 2000; Royal *et al.*, 2002a). Therefore broader selection goals have been introduced and are being continuously developed to include traits associated with longevity, for example fertility (Miglior *et al.*, 2005). Although many countries now have the opportunity to put selection pressure on fertility, the tools available to do so are not perfect. The challenge facing the dairy industry is therefore to regain a balance between milk yield and fertility by placing more emphasis on selection for fertility whilst still maintaining selection for milk yield.

Large physiological studies of dairy cows are expensive and for this reason uncommon. Therefore, the current chapter describes an analysis of a Danish dataset (see Chapter 3 for description of the dataset). Due to the similarities between the Danish and UK dairy cattle industries (with the exception of the breeds and numbers of cattle; see Chapter 1.2 for details) this was an interesting and useful preliminary investigation which is later compared to a UK dataset in Chapter 5.

The Danish dairy industry has published a fertility index since 1995 (Pedersen and Jensen, 1996). The current fertility index links information on several fertility measures

(days from first to last insemination in heifers and cows, days from calving to first insemination in cows, non return rate in heifers and cows, heat strength in heifers and cows and fertility treatments in cows) which are combined and weighted according to economic value (described in more detail Chapter 1). A high fertility index indicates better fertility. One limitation of all current fertility indices worldwide is that the traits are measured in the mature female and have low heritability so genetic progress in fertility is slow. The addition to fertility indices of an appropriate indicator trait for female fertility which is measurable in the juvenile male could increase the rate of genetic improvement in fertility.

Subsequent to work by Land (1973) who first proposed that sex linked characters in the female are expressed in the male, several studies in calves and lambs have examined potential physiological juvenile indicator traits for female reproduction (e.g. Haley *et al.*, 1989; Haley *et al.*, 1990; Mackinnon *et al.*, 1991; Royal *et al.*, 2000). These studies have focused on reproductive hormones (testosterone, luteinizing hormone), however to date and to the author's knowledge, no studies have investigated the potential of metabolic hormones as juvenile predictors at a genetic level.

Following parturition many cows enter a period of negative energy balance (NEB) which can last for several weeks (See Chapter 1 for details). Furthermore, the duration and severity of NEB postpartum, often determined by changes in body condition score (BCS), is unfavorably correlated (phenotypically and genetically) with the interval to first ovulation (Butler, 2000; de Vries & Veerkamp, 2000; Dechow *et al.*, 2002; Royal *et al.*,2002b). During this period of NEB, changes are seen in the concentrations of free fatty acids (FFA), glucose, growth hormone (GH), insulin, insulin-like growth factor 1 (IGF-1) and other regulatory hormones (Hart, 1983; Butler, 2000; Roche, 2000). Synthesis of GH by the anterior pituitary gland increases (Diskin *et al.*, 2003) causing an increase in lipolysis, which results in a raised amount of circulating FFA (Hart, 1983), of which some in turn is transported to the liver where they can accumulate and lead to liver ketosis (Bobe *et al.*, 2004). Furthermore, concentrations of circulating insulin,

glucose and liver GH receptors decrease, the latter of these causing IGF-1 production by the liver to decrease (Butler *et al.*, 2003).

It has been suggested that FFA, glucose, GH and insulin can be used as indicators of energy balance (Reist *et al.*, 2002), and thus also for NEB. However, in addition to their involvement in metabolic regulation, these metabolites and hormones *per se* have links with many aspects of reproduction including follicle growth and steroidogenesis (reviewed by Webb *et al.*, 2004; See Chapter 1 for details) such that altered concentrations during NEB can impair follicle growth and steroidogenesis (Roche, 2000). This therefore highlights the possible route for a genetic link between energy balance and fertility.

In order to be an efficient indirect indicator trait for female fertility, it is important that the parameter in question, such as a metabolite, has moderate heritability and sufficient genetic correlation with female fertility. To date, there have been no studies investigating the genetic relationship between metabolic regulation in calves and female fertility and few estimating the heritability of these possible indicators. Previous studies have found estimates for the heritability of GH ranging from $h^2 = 0.04$ to 0.60 (in 9 month old dairy calves; Løvendahl *et al.*, 1994; Sørensen *et al.*, 2002) and for glucose 0.41 (3-15 months of age dairy calves; Rowlands *et al.*, 1983).

The aim of this study was to estimate the genetic variation in FFA, glucose, GH and insulin plasma concentrations in 9-month old male dairy calves and assess the strength of any genetic link with the fertility of their female offspring.

4.2 MATERIALS AND METHODS

4.2.1 Animals and sampling

This study uses data from 1498 (269.5 days of age ± 11.1) male calves from a MOET breeding scheme. Calves were Danish Holstein (n = 1047), Danish Jersey (n = 200) and

Red Dane (n = 251) and were sampled, following an overnight fast, at 9 months of age (See Chapter 3 for details). Plasma samples were assayed for basal FFA, glucose, GH and insulin (See Chapter 2 for details). Fertility estimated breeding values (FertEBV), based on progeny test results on approximately 100 daughters per sire, were available for a subset (n = 810) of the male calves as adult sires (calculated by Danish Cattle Federation, Aarhus N, Denmark; See Chapter 3 for details).

4.2.2 Statistical analysis

Concentrations of GH, insulin and FFA were log-e transformed to give approximately normally distributed residuals. The geometric mean was obtained by back transformation (e^x) to give values in measured units.

A univariate mixed model was fitted to the data using the average information restricted maximum likelihood method in DMU software (Madsen & Jensen, 2002). Data for Danish Holstein, Red Dane and Danish Jersey were analysed jointly (ALL), in addition to a subset containing Danish Holsteins only (DH). The inclusion of various effects in the model was explored and the final model fitted to the hormone and metabolite data was:

$$Y_{ijklm} = \alpha + F_i + B_j + P_k + bD + A_l + S_m + \varepsilon_{ijklm}$$

Where:

Y_{ijklm} = the hormone or metabolite concentration

where fixed effects are:

α = intercept

F_i = farm (i = 1 to 4)

B_j = breed (j = 1 to 3)

P_k = protocol (Protocol 1 and 2)

D = age of animal in days

b = regression coefficient

and random effects are:

A_l = breeding value (N (0, σ_A^2**A**) where A is the numerator relationship matrix of animals available in the data)

S_m = experimental sampling day effect of sample (N (0, σ_B^2)

ε_{ijklm} = error term (N(0, σ_E^2)

Heritability (h^2) was calculated as the proportion of phenotypic variance (σ_p^2) attributable to additive genetic variance (σ_a^2) where the variance due to experimental sampling day effect (σ_b^2) was not included in the phenotypic variance:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2}$$

The standard error of the heritability estimates were calculated using the Taylor series expansion and the following formulae.

$$\frac{\partial h^2}{\partial \sigma_a^2} = \frac{\sigma_e^2}{(\sigma_a^2 + \sigma_e^2)^2} = A$$

$$\frac{\partial h^2}{\partial \sigma_e^2} = \frac{-\sigma_a^2}{(\sigma_a^2 + \sigma_e^2)^2} = B$$

$$\text{Var}(h^2) = [(A)^2 \times \text{Var}(\hat{\sigma}_a^2)] + [(B)^2 \times \text{Var}(\hat{\sigma}_e^2)] + [2 \times AB \times \text{Cov}(\hat{\sigma}_a^2\, \hat{\sigma}_e^2)]$$

$$\text{SE}(h^2) = \sqrt{\text{Var}(h^2)}$$

The significance of the fixed and random effects used in the model was assessed using SAS software (Version 8, SAS Institute Inc.) for each physiological trait analysed, but using a common final model as above. Approximate genetic correlations (r_A) were estimated by correlating the EBVs for the hormone or metabolite with the FertEBV.

4.3 RESULTS

4.3.1 Hormone and metabolite concentrations

The mean plasma concentration of FFA, glucose, GH and insulin for all breeds and for Danish Holsteins separately are shown in *Table 4.1*. The plasma concentration of FFA, glucose and insulin was slightly lower in Danish Holsteins compared to all breeds combined. The plasma concentration of GH was slightly higher in Danish Holsteins. However, overall there was little difference between the plasma hormone or metabolite concentrations in all breeds and Danish Holsteins. This is partly expected because Danish Holsteins make up more than half of the sampled animals however analysis of the Danish Holstein data separately does give further information.

Table 4.1 Number of calves sampled, mean, standard deviation (s.d.) and geometric mean of FFA concentrations (log-e mean; μeqv/l geometric mean), glucose (mmol/l mean), GH (log-e mean; ng/ml geometric mean) and insulin (log-e mean; pmol/l geometric mean) in Danish Holsteins (DH) and in all breeds combined (All).

Trait	N	Mean	s.d.	Geometric mean
DH-FFA	1007	6.26	0.28	524.77
ALL-FFA	1443	6.27	0.27	528.48
DH-Glucose	1003	4.83	0.49	---
ALL-Glucose	1438	4.87	0.50	---
DH-GH	1002	1.10	0.98	3.00
ALL-GH	1432	1.02	1.03	2.77
DH-Insulin	1004	3.42	0.62	30.68
ALL-Insulin	1431	3.44	0.62	31.19

4.3.2 Fixed effects

The significance of the fixed effects fitted in the univariate model are shown in *Tables 4.2 – 4.5*; farm and protocol are tested against the experimental sampling day

(denominator df = 110) whereas breed and age are tested against the animal (denominator df = 1325). The least square means, calculated to give an indication of the effect attributable to the different levels of the fixed effects, are shown in *Figures 4.4 – 4.7* for each hormone or metabolite. The effects of station, breed and protocol were significant (P<0.05) in each analysis. Although the regression of age was not significantly different to zero, it was decided that it should remain in the model due to the monthly sampling leading to a spread in the ages of the animals (220-313 days).

Table 4.2 Significance of effects in the model for FFA analysis (df = degrees of freedom, NS = non significant P>0.05).

Effect	Numerator df	Denominator df	F value	Significance
Farm	3	110	13.25	P<0.0001
Breed	2	1325	8.31	P=0.0003
Protocol	1	110	25.56	P<0.0001
Age	1	1325	0.18	NS

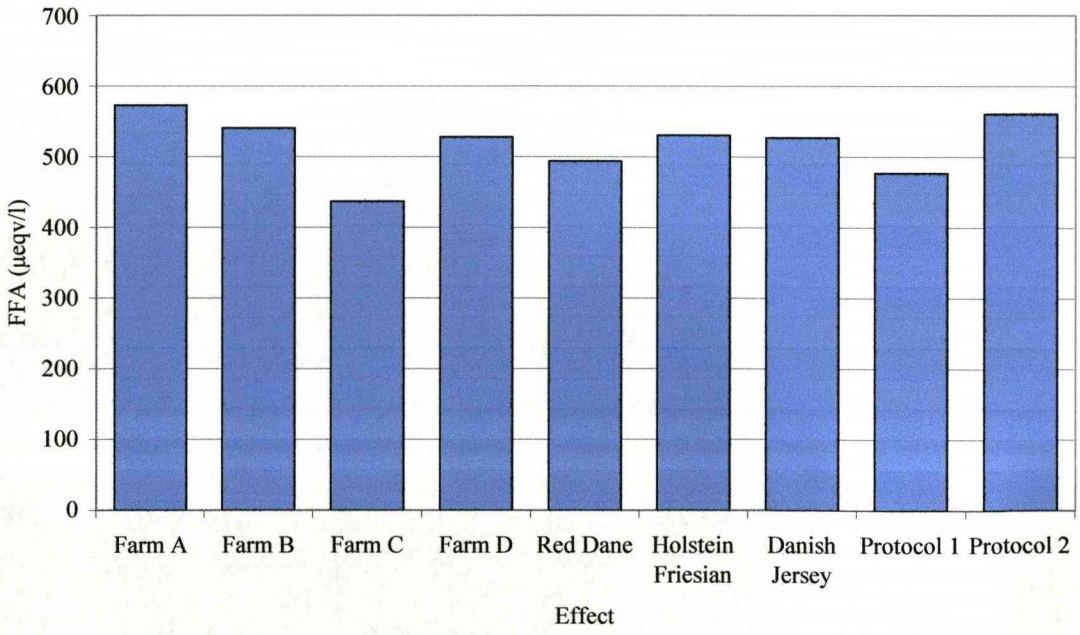

Figure 4.1 The least square means for FFA (μeqv/l) for the different levels of the fixed effects in the model.

Table 4.3 Significance of effects in the model for glucose analysis (df = degrees of freedom, NS = non significant P>0.05).

Effect	Numerator df	Denominator df	F value	Significance
Farm	3	110	12.73	P<0.0001
Breed	2	1320	8.24	P=0.0003
Protocol	1	110	4.86	P=0.0296
Age	1	1320	3.26	NS

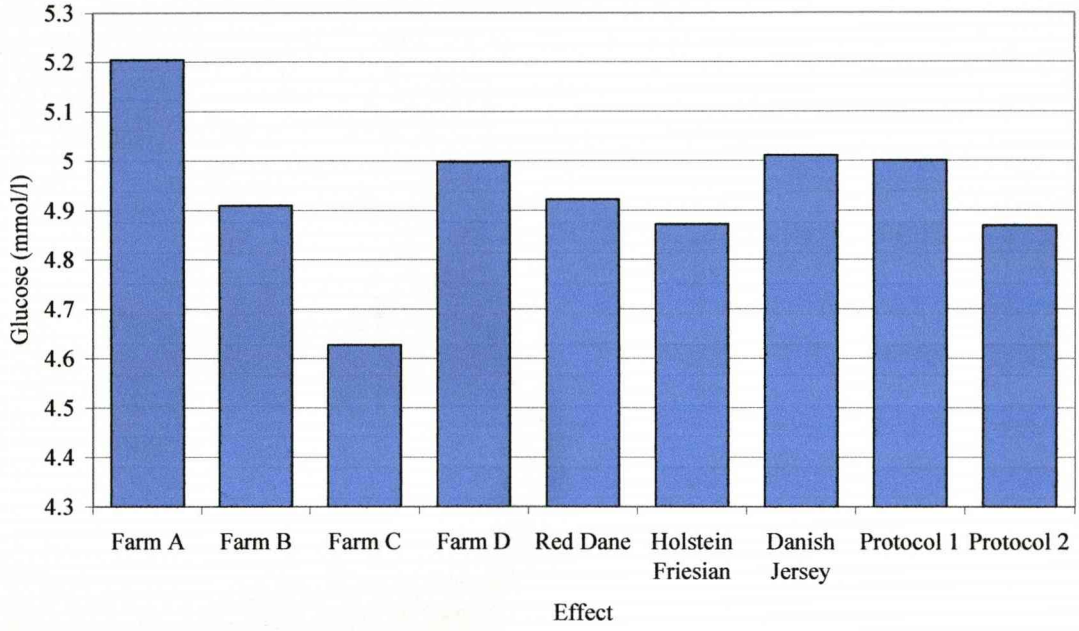

Figure 4.2 The least square means for glucose (mmol/l) for the different levels of the fixed effects in the model.

Table 4.4 Significance of effects in the model for GH analysis (df = degrees of freedom, NS = non significant P>0.05).

Effect	Numerator df	Denominator df	F value	Significance
Farm	3	108	13.20	P<0.0001
Breed	2	1316	17.92	P<0.0001
Protocol	1	108	78.17	P<0.0001
Age	1	1316	1.29	NS

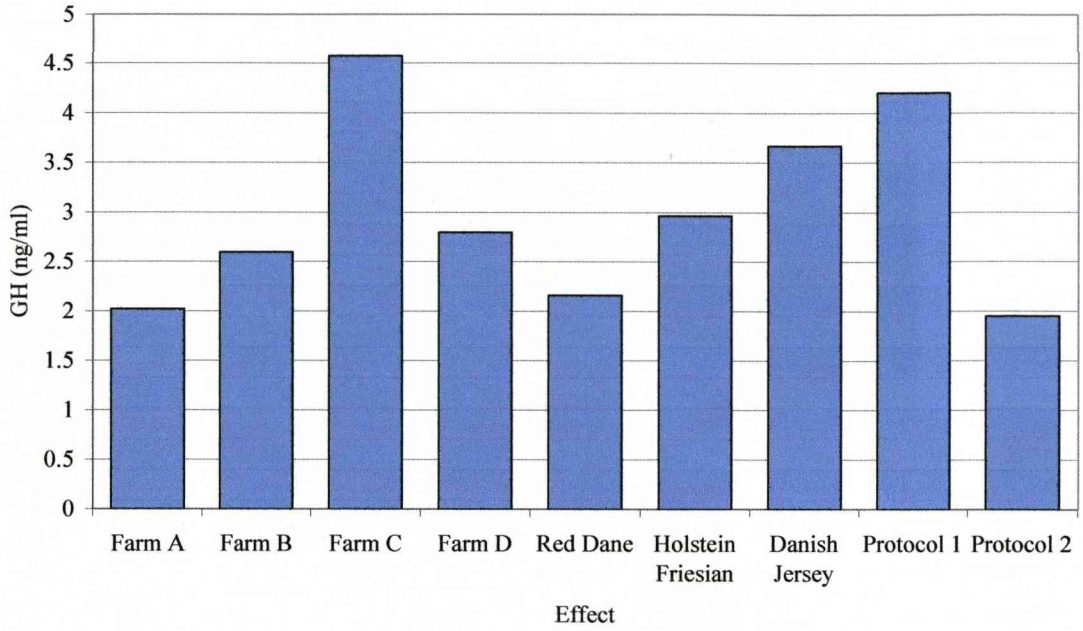

Figure 4.3 The least square means for GH (ng/ml) for the different levels of the fixed effects in the model.

Table 4.5 Significance of effects in the model for insulin analysis (df = degrees of freedom, NS = non significant P>0.05).

Effect	**Numerator df**	**Denominator df**	**F value**	**Significance**
Farm	3	109	8.53	P<0.0001
Breed	2	1314	8.83	P=0.0002
Protocol	1	109	9.27	P=0.0029
Age	1	1314	1.06	NS

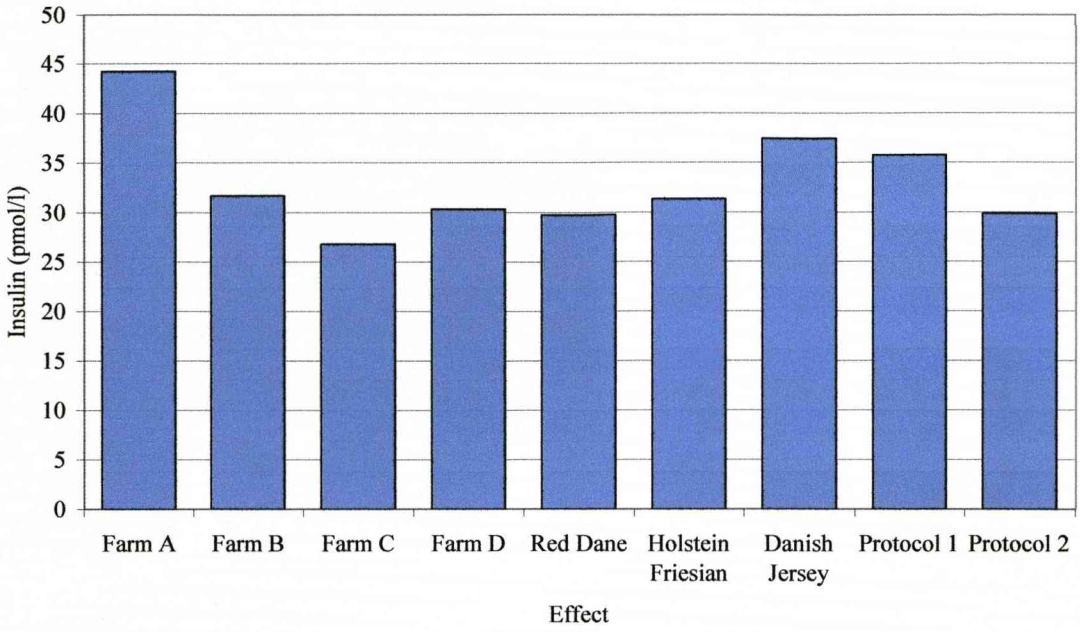

Figure 4.4 The least square means for insulin (pmol/l) for the different levels of the fixed effects in the model.

4.3.3 Heritability

The heritability estimates plus their standard errors are shown in *Table 4.6*. Free fatty acids had a moderate heritability in both DH and ALL (0.15 ± 0.06; 0.11 ± 0.05). Similarly, the heritability for both glucose and insulin was moderate in both DH and ALL (0.15 ± 0.07; 0.27 ± 0.06 and 0.16 ± 0.06; 0.21 ± 0.06, respectively). However, the heritability estimate for GH was low in both DH and in ALL (0.04 ± 0.04; 0.09 ± 0.05). In general the heritability estimates for glucose, GH and insulin were higher in ALL than in DH, although not significantly so. The standard errors were of similar magnitude in both cohorts. The effects of station, breed and protocol were significant ($P<0.05$) in each analysis.

Table 4.6 Estimated heritability for plasma FFA, glucose, GH and insulin plus standard errors (s.e.)

Trait	n	h^2	s.e. (h^2)
DH-FFA	1007	0.15	0.06
ALL-FFA	1443	0.11	0.05
DH-Glucose	1003	0.15	0.07
ALL-Glucose	1438	0.27	0.06
DH-GH	1002	0.04	0.04
ALL-GH	1432	0.09	0.05
DH-Insulin	1004	0.16	0.06
ALL-Insulin	1431	0.21	0.06

4.3.4 Correlations between fertility and metabolic indicator traits

Genetic correlations of FertEBV with the hormone and metabolite data are shown in *Table 4.8*. Correlations of FertEBV with glucose and FFA EBVs were negative and significant in ALL. In DH the genetic correlation of FertEBV with FFA EBV was negative and significant. This indicates that on average in this study, male calves with lower than average glucose or FFA at 9 months of age have female offspring with better than average fertility (a shorter interval to first artificial insemination postpartum and shorter interval between first and last insemination). Estimated genetic correlations of FertEBV with GH and insulin were small, inconsistent and non-significant in DH and ALL.

Table 4.7 Correlations between estimated breeding values (r_A) for fertility (FertEBV) and FFA, glucose, GH and insulin (NS = non significant P>0.05).

Trait	N	r_A	Significance
DH-FFA	534	-0.19	P<0.0005
ALL-FFA	812	-0.15	P<0.0005
DH-Glucose	533	-0.06	NS
ALL-Glucose	810	-0.10	P<0.005
DH-GH	533	0.04	NS
ALL-GH	813	-0.02	NS
DH-Insulin	535	-0.02	NS
ALL-Insulin	812	-0.01	NS

4.4 DISCUSSION

The principal findings in this study are as follows. A considerable amount of the phenotypic variance seen in FFA, glucose and insulin appears to be genetic. The approximate genetic correlations of FertEBV with FFA and glucose were negative and significant. The heritability of GH was low and GH and insulin showed no genetic correlation with FertEBV.

4.4.1 Heritability

The heritability of GH concentrations was low which is in agreement with others (Løvendahl *et al.*, 1994; Grochowska *et al.*, 2001). This may be due to pulsatile GH secretion. Large secretory pulses of GH add noise to the data and thereby to the total variance and this reduces the heritability estimate (Theilgaard *et al.*, 2007). This could partly be overcome by serial blood sampling over a long period. Alternatively the stimulated release of GH is studied. This is more cost and time effective as fewer blood samples, over a shorter period of time are needed. Grochowska *et al.* (2001) estimated the genetic variation in peak GH release following thyrotropin-releasing hormone challenge and found a higher heritability than for baseline GH (Polish Friesian male and

female n= 214, age 335 ± 8 d; h^2 = 0.14 ± 0.11 versus h^2 = 0.02 ± 0.11). Similarly Løvendahl *et al.* (1994) found a higher heritability in peak GH following growth hormone releasing factor stimulation (Danish Jersey, Red Dane, Danish Friesian and Danish Red and White male n = 284 and female n = 272, age 242-311d; h^2 = 0.42 ± 0.16 males, h^2 = 0.60 ± 0.16 females) rather than for baseline GH (h^2 = 0.04 ± 0.12 males, h^2= 0.60 ± 0.16 females).

This study has shown that the concentrations of FFA, glucose and insulin in male calves are moderately heritable. This is to some extent due to these being easier to study, as concentrations are more stable leading to less "noise" in the data. The heritability estimate for glucose is however lower than in a previous study by Rowlands *et al.* (1983), which examined the genetic variation in glucose and other circulating metabolites in fed young British Friesian bulls (n = 428; 3-15 months of age). The heritability estimate for plasma glucose in their study was high (h^2 = 0.41 ± 0.17) although the standard error was large. Heritability estimates for glucose, similar to those in the present study, were reported in a Danish study in Red Dane, Danish Friesian, Danish Jersey and Danish Red and White 9-month old calves (h^2 = 0.22 ± 0.08 males n = 451, h^2 = 0.28 ± 0.09 females n = 371; Løvendahl and Jensen, 1997). The reported heritability estimates for insulin and FFA were higher than found in the present study (h^2 = 0.04 ± 0.08 males n = 334, h^2 = 0.43 ± 0.11 females n = 300; h^2 = 0.52 ± 0.16 males n = 198, h^2 = 0.32 ± 0.14 females n = 190, respectively; Løvendahl and Jensen, 1997) although the number of calves was low.

The heritability estimates found here are encouraging because in order to be used as indirect selection criteria, in addition to having a genetic correlation to the trait of interest, in this case fertility, the criterion must have a moderate heritability (Falconer & Mackay, 1996b). Although GH had low genetic variation, FFA and glucose showed sufficient genetic variation to be suitable, from a heritability point of view, as an indirect selection criterion.

4.4.2 Hormones and metabolites

Plasma concentrations of FFA, glucose, GH and insulin found in this study were comparable to those found in similar aged animals during mild feed deprivation (e.g. Løvendahl *et al.*, 1994; Govoni *et al.*, 2003; Taylor *et al.*, 2004). The plasma concentration of FFA increases during a fast with a significant increase seen after just one night of fasting (Sinnett-Smith *et al.*, 1987). Although in the present study, pre-fast concentrations of FFA were not available, when compared to pre-fast concentrations in previous studies (Sinnett-Smith *et al.*, 1987; Woolliams *et al.*, 1992; Jorritsma *et al.*, 2003) the FFA concentrations in the present study were significantly elevated after one night of fasting.

Plasma glucose concentrations in ruminants are more stable than in monogastric species with significant reduction seen after 24-48 hours of fasting (Diskin *et al.*, 2003; Chelikani *et al.*, 2004). In the present study the concentrations of glucose were similar to those seen in fed animals of other studies. Insulin falls in response to reduced glucose therefore since glucose concentrations appear not to have dropped; we propose that insulin concentration in these animals will also not have dropped significantly. Growth hormone concentration shows greater variation and release tends to increase during the night and periods of energy restriction (Tannenbaum, 1988). The plasma concentration of GH in this study (2.69 ng/ml DH; 2.56 ng/ml ALL) are similar to those reported by Chelikani *et al.* (2004) after 12-24 hours fast (1.50-1.80 ng/ml).

4.4.3 Approximate genetic correlations with fertility EBV

Growth hormone and insulin showed no significant genetic correlation with FertEBV. The genetic correlations of FertEBV with FFA were negative and significant in all breeds combined and in the Danish Holsteins subset. Furthermore, the genetic correlation of FertEBV with glucose was negative in both the Danish Holstein subset and all breeds, although this estimate was not significantly different from zero in the Danish Holsteins. Therefore, on average, male calves with high glucose and FFA

following overnight fast at 9 months of age tend to produce female offspring with reduced fertility. The concentration of FFA following a fast indicates the extent to which an animal breaks down stored fat (lipolysis) during energy shortage. This correlation potentially indicates that calves that mobilise a large amount of FFA in response to a fast tend to have female offspring with reduced fertility. The plasma concentration of FFA, in addition to BCS, is a good indicator of the metabolic status of an animal and raised concentrations of FFA are seen during NEB (Reist *et al.*, 2002). In the past cows have been selected for "dairy type" which is primarily a lower BCS and high milk yield. The length and severity of NEB is unfavourably correlated (phenotypically and genetically) to the interval to first ovulation postpartum (Butler, 2000; de Vries & Veerkamp, 2000; Dechow *et al.*, 2002) so selection in the past for yield and "dairy type" may have inadvertently been for the animals that have low BCS and experience severe or prolonged NEB.

In this study, a short period of fasting may have resulted in lowered glucose concentrations. It is unlikely that the drop will be significant, however, there was clearly variation in the glucose concentration due possibly to differing response to short term feed deprivation. The negative genetic correlation in this study between FertEBV and glucose would indicate that calves whose plasma glucose drops by a greater extent or is at a lower concentration, tend to have female offspring with improved fertility, whereas calves whose plasma glucose drops to a lesser extent or is at a higher concentration, tend to have female offspring with lower fertility. Blood glucose concentration is maintained within a narrow range by the alternating release of glucagon, when glucose drops below a certain concentration and insulin, when glucose rises above a certain concentration (Jiang and Zhang, 2003). It may be possible that in this study calves whose glucose concentration dropped the most following the overnight fast have a lower critical concentration below which the release of glucagon is stimulated. Consequently in these animals the plasma concentration of glucose is able to drop further before glucagon is released and both gluconeogenesis and glycogenolysis are initiated. The relationship between this in the male calf and having female offspring with better fertility is however unclear.

It is likely that the concentrations of FFA and glucose at 9 months of age are genetically the same trait in males and females (Løvendahl and Jensen, 1997). Therefore it is expected that female offspring from the males with high FFA and glucose after overnight fast may also have a tendency to mobilise body reserves quickly during energy shortage which may lead to a cow that will use most of her body reserves for milk production, go into extreme NEB and subsequently have fertility problems such as a prolonged interval to first ovulation postpartum. Calves which are better at withholding their body reserves may go on to be cows that experience less severe NEB and less subsequent fertility problems. This relationship between metabolic regulation in bull calves with their offspring's fertility needs further investigation before use by the dairy breeding industry. Correlated responses to selection in other traits would need to be determined to prevent undesirable side affects to selection.

4.4.4 Future work

This analysis has provided evidence to show that additive genetic variance is responsible for a substantial proportion of the phenotypic variation in a number of hormones in Danish male calves. Furthermore, these results indicate that glucose and FFA are negatively correlated to fertility in their female offspring. Glucose and FFA may therefore be of potential interest to dairy cattle selection programmes to improve female fertility, as a measurement in young bulls. This relationship between metabolic regulation in bull calves with their offspring's fertility needs further investigation before use by the dairy industry. Correlated responses to selection in other traits would need to be determined to prevent undesirable side affects to selection. This genetic correlation has been found in a Danish cattle population, in an experiment sampling calves after an overnight fast and following a certain protocol, and may also be present in a UK cattle population. This is the subject of Chapter 5.

Chapter 5: GENETIC VARIATION OF METABOLITES AND HORMONES IN UK HOLSTEIN-FRIESIAN CALVES

5.1 INTRODUCTION

The UK dairy cattle industry faces similar challenges to the Danish and indeed the worldwide dairy industry (See Chapter 1 and Chapter 4). Common problems include poor fertility and although many countries now publish fertility indices they are far from perfect. It is hoped that the recently introduced UK fertility index will help slow the decline by providing the opportunity to select for female fertility (Wall *et al.*, 2003a; See Chapter 1). The UK fertility index is based on calving interval and non-return rate (56 days after insemination; NR56) predicted transmitting abilities (PTAs) weighted according to their relative economic values. To estimate calving interval and NR56 PTAs, six traits are analysed together: calving interval, NR56, interval to first service, number of inseminations per conception, body condition score (BCS), and milk yield, the latter two because of their genetic correlation to fertility and their higher heritability ($h^2 \pm$ standard error, 0.033 ± 0.001 n = 32205; 0.018 ± 0.001 n = 42995; 0.035 ± 0.001 n = 43029; 0.020 ± 0.002 n = 32209; 0.237 ± 0.008 n = 12866; 0.329 ± 0.003 n = 43029 respectively, Wall *et al.*, 2003b). It is not possible yet to assess the effects of this index, however it does have limitations in that the six traits used to estimate calving interval and NR56 PTA are measured in the mature female at some point between lactation one and two, at approximately two to three years of age. Secondly, the four fertility traits have low heritability (heritability range 0.018 to 0.035, Wall *et al.*, 2003b). The addition of an indicator trait to the fertility index, that is measurable in the juvenile male, is heritable and genetically correlated to female fertility, could potentially improve the rate of genetic improvement in fertility.

As described in Chapter 4 the duration and severity of negative energy balance (NEB) postpartum is unfavourably genetically correlated to fertility (Butler, 2000; Dechow *et al.*, 2002; de Vries & Veerkamp, 2000; Royal *et al.*, 2002). During this period, changes are seen in the concentrations of free fatty acids (FFA), glucose, growth hormone (GH),

insulin, insulin like growth factor 1 (IGF-1) and other regulatory hormones (Butler, 2000; Hart, 1983; Spicer *et al.*, 1990; Roche, 2000; See Chapter 1 for details). These hormones and metabolites *per se* have links with many aspects of reproduction (See Chapter 4 for details).

The initial requirement for an indirect selection criterion is moderate heritability. Analysis of a Danish dataset of nine-month old Danish Jersey, Red Dane and Danish Holstein male calves showed some promising results (see Chapter 4), providing evidence for the presence of genetic variation in the concentration of FFA, glucose and insulin. The aim of the current chapter is to further this analysis in a UK dataset by assessing the genetic variation present in FFA, glucose, GH, insulin and IGF-I concentrations in Holstein-Friesian male and female dairy calves (approximately four months of age).

5.2 MATERIALS AND METHODS

Detailed descriptions of assay techniques and statistics, database construction and content are outlined in Chapters 2 and 3.

5.2.1 Animals and sampling

The analyses described herein use Datasets 1 (D1) and 2 (D2) described in detail in Chapter 3. Plasma samples were collected from Holstein-Friesian calves (average age days ± standard deviation; 126 ± 12.7 d) during two studies: Dataset 1 (D1-F, n = 326 females, D1-M, n = 256 males; 2002-2006; MOET breeding scheme) and Dataset 2 (D2-F, n = 496 females; 1996-2001; 7 commercial dairy herds; *Table 5.1*). Plasma samples were analysed for FFA, glucose, GH, insulin and total IGF-I (See Chapter 2).

Table 5.1 Number and age (days ± standard deviation (s.d.)) of calves of each sex from each dataset sampled.

		Number	**Mean age ± s.d. /days**
Dataset 1	Males	256	127.1 ± 11.0
	Females	320	120.2 ± 16.9
Dataset 2	Females	501	129.2 ± 8.3
Total		1077	126.0 ± 12.7

5.2.2 Statistical analysis

5.2.2.1 Analysis of the hormone and metabolite data

Several blood samples were taken during the day of sampling and selected ones were used for the measurement of FFA, glucose, GH, insulin and IGF-1 (See Chapter 3). The hormone and metabolite data was checked for normality (*Figures 5.1* to *5.5*). Concentrations of FFA, GH, insulin and IGF-I were $\log_{10}$ transformed to give approximately normally distributed residuals. The distribution of glucose concentrations was approximately normal therefore no transformation was necessary (*Figure 5.1*).

Univariate mixed models were fitted to the hormone and metabolite data using ASREML software (Gilmour *et al.* 2006). To investigate the possibility of combining the three subsets of data (D1-M, D1-F and D2-F) they were first analysed separately. The inclusion of weight at testing in the model was investigated. However, because weight has a significant genetic component itself, subsequent bivariate analyses were carried out with weight to ensure that genetic variation in the hormone or metabolite, highlighted by univariate analysis, was not in fact attributable to weight. Genetic variation in the hormone or metabolite was similar in both univariate and bivariate analysis and thus weight was not fitted as an effect in the univariate model. The inclusion of various other components in the model was explored. The fixed effects in the initial model were sire percentage Holstein, age and the experimental batch. The genetic relationships were modelled by the relationship matrix calculated from the three-

generation pedigree (over 4600 animals). The initial univariate model fitted to the hormone and metabolite data for each subset was:

$$Y_{ij} = \alpha + {}_{b1}P + {}_{b2}D + B_i + A_j + \varepsilon_{ij}$$

Where:

Y_{ij} = the hormone or metabolite concentration

the fixed effects are:

α = intercept

${}_{b1}P$ = regression variable of sire percentage Holstein with coefficient *b1*

${}_{b2}D$ = regression variable of age of animal in days with coefficient *b2*

B_i = the fixed effect of experimental batch (i = 1 to 131)

and the random effects are:

A_j = breeding value (N (0, σ_A^2**A**) where A is the numerator relationship matrix of animals available in the data)

ε_{ij} = error term (N (0, σ_E^2)

From these analyses the mean concentration, genetic variance plus its standard error and the residual variance plus its standard error for each subset (D1-M, D1-F and D2-F) for each hormone or metabolite was obtained (*Table 5.2*). Within each hormone or metabolite the genetic variance for each of the three subsets was similar and in addition to this each one had a small standard error. However the difference between the residual variance within each trait (for subsets D1-M, D1-F and D2-F) was sizeable, the small standard errors being suggestive of significant differences; had the standard errors been large the confidence in the estimates would have been reduced (*Table 5.2*). It appeared that in most cases the residual variance in the D1-F subset was greater whilst the residual variance in D2-F was smallest. A possible explanation for this is that because D2-F was distributed over 7 privately owned commercial herds and the experimental procedure was tightly controlled whereas D1-M and D1-F were conducted at two young stock units for a large breeding company. In the case of D1-F, calves were transported from nearby rearing units to the testing station prior to sampling. The protocol required that this be done 1 to 2 hours prior to sampling to allow the calves time to adjust to the environment. However on some occasions the calves were transported just before sampling which

could increase "stress" in these animals and create more environmental "noise" in the measurements.

Table 5.2 Mean concentration (FFA, GH, insulin & IGF-1 in log_{10} units, glucose in mmol/l), genetic variance plus its standard error (s.e) and the residual variance plus its standard error for each subset (D1-M, D1-F and D2-F)

Trait	Dataset	Mean	$\sigma^2_A \pm$ s.e	$\sigma^2_E \pm$ s.e
Glucose	D1-M	5.09	0.0878 ± 0.07	0.2447 ± 0.05
	D1-F	4.72	0.0135 ± 0.05	0.4854 ± 0.06
	D2-F	4.26	0.1234 ± 0.06	0.1718 ± 0.05
FFA	D1-M	5.40	0.0269 ± 0.02	0.1191 ± 0.02
	D1-F	4.68	0.0148 ± 0.02	0.1628 ± 0.02
	D2-F	5.47	0.0174 ± 0.01	0.0497 ± 0.01
GH	D1-M	1.58	0.0356 ± 0.08	0.5193 ± 0.08
	D1-F	1.07	0.0804 ± 0.06	0.3011 ± 0.05
	D2-F	0.72	0.0705 ± 0.07	0.3961 ± 0.07
Insulin	D1-M	3.41	0.0573 ± 0.03	0.1081 ± 0.03
	D1-F	3.69	0.0000 ± 0.00	0.2826 ± 0.02
	D2-F	3.04	0.0683 ± 0.06	0.2930 ± 0.05
IGF-1	D1-M	5.47	0.0722 ± 0.03	0.0433 ± 0.02
	D1-F	4.61	0.0682 ± 0.05	0.2876 ± 0.05
	D2-F	4.94	0.1011 ± 0.03	0.0545 ± 0.03

To allow the analysis of the combined data (D1-M + D1-F + D2-F) extra random terms were fitted to account for additional residual variance in subset D1-M, D1-F and D2-F where necessary. The additional random terms were added for the two subsets with the highest residual variance within each trait. The inclusion of various components in the model was explored. The fixed effects in the final model were sire percentage Holstein, age, experimental batch, sex and sex by batch interaction.

Hence the univariate model fitted to the combined datasets (D1-M + D1-F + D2-F) for each hormone and metabolite was:

$$Y_{ijklm} = \alpha + {}_{b1}P + {}_{b2}D + B_i + S_j + S.B + R1_k + R2_l + A_m + \varepsilon_{ijklm}$$

Where:

Y_{ijklm} = the hormone or metabolite concentration

the fixed effects are:

α = intercept

${}_{b1}P$ = regression variable of sire percentage Holstein with coefficient *b1*

${}_{b2}D$ = regression variable of age of animal in days with coefficient *b2*

B_i = the fixed effect of experimental batch (i = 1 to 131)

S_j = sex (j = female or male)

S.B = the sex by batch interaction

and random effects are:

$R1_k$ = the extra random term for extra residual variance (in subset D1-M, D1-F or D2-F)

$R2_l$ = the extra random term for extra residual variance (in subset D1-M, D1-F or D2-F)

A_m = breeding value (N $(0, \sigma_A^2\mathbf{A})$ where A is the numerator relationship matrix of animals available in the data)

ε_{ijklm} = error term (N $(0, \sigma_E^2)$)

Three heritability estimates, one each for subset D1-M, D1-F & D2-F, were calculated for each hormone or metabolite. The variance components included in the phenotypic variance depended on which extra random terms were fitted. For example, the univariate model fitted for glucose contained extra random terms for D1-M and D1-F because out of the three subsets these had the highest residual variance. Therefore four components of variance were estimated:

D1-M extra variance	(a)
D1-F extra variance	(b)
Additive genetic variance	(c)
Residual variance	(d)

The three heritability estimates were calculated as follows:

$$\text{D1-M} \quad h^2 = \frac{c}{a + c + d}$$

$$\text{D1-F} \quad h^2 = \frac{c}{b + c + d}$$

$$\text{D2-F} \quad h^2 = \frac{c}{c + d}$$

5.2.2.2 Bivariate analysis of hormone or metabolite data with each other and with weight

Bivariate analysis of the hormone and metabolite data with each other was also carried out to assess the strength of genetic and phenotypic relationships between parameters. The fixed and random terms were the same as those for the univariate analyses. Additional random terms were fitted to account for extra residual variance in two of the three datasets (D1-M, D1-F and D2-F). Therefore in each analysis two extra random terms were fitted for trait one and for trait two. The bivariate model fitted to the data was as follows:

$$Y_{ijklo} \,\&\, Z_{ijmno} = \alpha + {}_{b1}P + {}_{b2}D + B_j + S_i + S.B + R1_k + R2_l + R3_m + R4_n + A_o + \varepsilon_{ijklmno}$$

Where:

Y_{ijklo} = the hormone or metabolite concentration 1

Z_{ijmno} = the hormone or metabolite concentration 2

the fixed effects in common to both Y and Z are:

α = intercept

${}_{b1}P$ = regression variable of sire percentage Holstein with coefficient *b1*

${}_{b2}D$ = regression variable of age of animal in days with coefficient *b2*

B_i = the fixed effect of experimental batch (i = 1 to 74)

S_j = sex (j = female or male)

S.B = the sex by batch interaction

the random effects applicable to Y only are:

$R1_k$ = the extra random term for extra residual variance

$R2_l$ = the extra random term for extra residual variance

the random effects applicable to Z only are:

$R3_m$ = the extra random term for extra residual variance

$R4_n$ = the extra random term for extra residual variance

and the random effects common to both Y and Z are:

A_o = breeding value ($N(0, \sigma_A^2\mathbf{A})$ where A is the numerator relationship matrix of animals available in the data)

$\varepsilon_{ijklmno}$ = error term ($N(0, \sigma_E^2)$)

Univariate analysis of weight of the animal on the day of blood sampling was carried out initially to establish whether additional residual variance components needed to be fit for the different datasets and to determine the fixed effects to be fitted. The initial univariate model fitted to weight was:

$$Y_{ij} = \alpha + {}_{b1}P + {}_{b2}D + B_i + F.D + A_j + \varepsilon_{ij}$$

Where:

Y_{ij} = the weight

the fixed effects are:

α = intercept

${}_{b1}P$ = regression variable of sire percentage Holstein with coefficient *b1*

${}_{b2}D$ = regression variable of age of animal in days with coefficient *b2*

B_i = the fixed effect of experimental batch (i = 1 to 131)

F.D = the farm by age interaction

and the random effects are:

A_j = breeding value ($N(0, \sigma_A^2\mathbf{A})$ where A is the numerator relationship matrix of animals available in the data)

ε_{ij} = error term ($N(0, \sigma_E^2)$)

From this analysis the mean weight, genetic and residual variance (± standard error) for each subset (D1-M, D1-F and D2-F) was obtained (*Table 5.3*). From *Table 5.3* it was decided that, in line with the univariate analyses of the hormones or metabolites, extra

random terms would be fitted to account for additional residual variance in D1-M and D1-F.

Table 5.3 Mean weight (kg), genetic variance (± standard error) and the residual variance (± standard error) for each subset (D1-M, D1-F and D2-F)

Trait	Dataset	Mean	$\sigma^2_A \pm$ s.e.	$\sigma^2_E \pm$ s.e.
Weight	D1-M	150.84	0.6421 ± 0.51	1.7614 ± 0.44
	D1-F	122.28	0.8395 ± 0.65	2.2350 ± 0.45
	D2-F	119.81	0.7720 ± 0.32	0.9447 ± 0.27

The bivariate analysis of the hormone or metabolite with weight was then carried out. The fixed and random terms for the hormone or metabolite were the same as those for the univariate analysis. The inclusion of various terms in the weight analysis was investigated and is shown below. Although, when fitting an interaction term the single terms must be fit separately in the model as well, in the case of the farm by age interaction the effect of farm is already accounted for in the experimental batch term and therefore it was not fit separately. Additional random terms were fitted to account for extra residual variance in two of the three datasets (D1-M, D1-F and D2-F). Therefore in each analysis two extra random terms were fitted for trait one and for trait two. The bivariate model fitted to the data was as follows:

$$Y_{ijklo} \,\&\, Z_{ijmno} = \alpha + {}_{b1}P + {}_{b2}D + B_i + S_j + S.B + S.D + F.D + R1_k + R2_l + R3_m + R4_n + A_o + \varepsilon_{ijklmno}$$

Where:

Y_{ijklo} = the hormone or metabolite concentration

Z_{ijmno} = the weight

the fixed effects in common to both Y and Z are:

α = intercept

${}_{b1}P$ = regression variable of sire percentage Holstein with coefficient *b1*

${}_{b2}D$ = regression variable of age of animal in days with coefficient *b2*

B_i = the fixed effect of experimental batch (i = 1 to 74)

S_j = sex (j = female or male)

S.B = the sex by batch interaction

the fixed effects applicable to Z (weight) only are:

S.D = the sex by age interaction

F.D = the farm by age interaction

the random effects applicable to Y only are:

$R1_k$ = the extra random term for extra residual variance

$R2_l$ = the extra random term for extra residual variance

the random effects applicable to Z only are:

$R3_m$ = the extra random term for extra residual variance

$R4_n$ = the extra random term for extra residual variance

and the random effects common to both Y and Z are:

A_o = breeding value ($N(0, \sigma_A^2\mathbf{A})$ where A is the numerator relationship matrix of animals available in the data)

$\varepsilon_{ijklmno}$ = error term ($N(0, \sigma_E^2)$)

The analysis of the hormone and metabolites with each other and with weight gave three heritability estimates (D1-M, D1-F and D2-F) for each bivariate analysis (15 in total). Since the additional random variance terms, to account for differences in the residual variance in each subset, only affected the size of the phenotypic variance for each hormone and weight there were three phenotypic correlation estimates for each bivariate analysis (one for each subset of data) and just one genetic correlation estimate.

5.2.2.3 The significance of fixed and random effects

The standard error of the heritability estimates were calculated using ASREML software (Gilmour *et al.,* 2006). The significance of the fixed and random effects used in the model were assessed using Wald F statistics and the likelihood ratio test respectively. The log likelihood values are obtained from ASREML and the test statistic D approximates to the chi-squared distribution with 1 degree of freedom. It is calculated by the following equation:

$D = -2\,[\log(R2) - \log(R1)]$

Where:

R1 = the log likelihood for the full model

R2 = the log likelihood for the restricted model i.e. with the random term in question removed

The significance of the genetic correlations were assessed through comparing the log likelihood of the full model to the log likelihood when the genetic covariance is fixed at zero. The D statistic is then calculated and approximates to the chi-squared distribution with one degree of freedom (as above).

The Wald F statistics produced by ASREML should be interpreted with caution. ASREML advises users that "the aim of the conditional Wald statistic is to facilitate inference for fixed effects and it is not meant to be prescriptive nor is it foolproof for every setting" (Gilmour *et al.* 2006). Furthermore it is not possible to calculate the denominator degrees of freedom and so these are assumed to be large (approximately infinity) when analysing large datasets (n = >1000). For these reasons it is advised to use the conditional Wald F significance test to give an indication of the significance of fixed effects but particularly if terms are almost significant to leave them in the model.

5.3 RESULTS

5.3.1 Hormone and metabolite concentrations

The number of animals, mean plasma concentration in measured units (before fitting the model), standard deviation and standard error of glucose, FFA, GH, insulin and IGF-I for each dataset are shown in *Table 5.4*.

Table 5.4 Number of calves sampled, mean in measured units, standard deviation (S.D.) and standard error of the mean (S.E.) of glucose (mmol/l), FFA (μeqv/l), GH (ng/ml), insulin (pmol/l), IGF-I (ng/ml), weight (kg) and age (days) in each subset (D1-M, D1-F and D2-F) and in the total females (D1-F + D2-F).

Trait	Dataset	Number	Mean	S.D.	S.E.
Glucose	D1-M	256	5.10	0.75	0.05
	D1-F	320	4.73	0.87	0.05
	D2-F	493	4.26	0.83	0.04
	D1-F+D2-F	813	4.45	0.88	0.03
FFA	D1-M	256	261.97	155.66	9.73
	D1-F	320	126.77	92.87	5.19
	D2-F	493	261.02	122.67	5.52
	D1-F+D2-F	813	208.18	129.67	4.55
GH	D1-M	256	6.33	5.22	0.33
	D1-F	320	3.66	2.87	0.16
	D2-F	498	2.69	2.21	0.10
	D1-F+D2-F	818	3.07	2.53	0.09
Insulin	D1-M	256	38.88	30.19	1.89
	D1-F	320	50.09	35.67	1.99
	D2-F	498	30.07	31.72	1.42
	D1-F+D2-F	818	37.90	34.70	1.21
IGF-1	D1-M	254	263.51	110.34	6.92
	D1-F	317	126.13	78.65	4.42
	D2-F	489	159.98	75.52	3.42
	D1-F+D2-F	806	146.67	78.48	2.76
Weight	D1-M	238	150.84	24.14	1.56
	D1-F	306	122.28	22.52	1.29
	D2-F	495	119.81	19.66	0.88
	D1-F+D2-F	801	120.75	20.82	0.74
Age	D1-M	256	127.07	10.96	0.68

	D1-F	326	120.38	16.80	0.93
	D2-F	498	129.32	8.48	0.38
	D1-F+D2-F	824	125.78	13.19	0.46

Figures 5.1 – 5.5 show the distribution of plasma concentrations of glucose, FFA, GH, insulin and IGF-I in the calves sampled. *Figure 5.6* highlights the difference in weight between males and females sampled. It is evident that concentrations of glucose, GH and IGF-I tended to be higher in males than in females (*Figures 5.1 – 5.5*); this is probably due to males having a higher growth rate and reaching a higher weight at the age of sampling compared to the females (*Figure 5.6*).

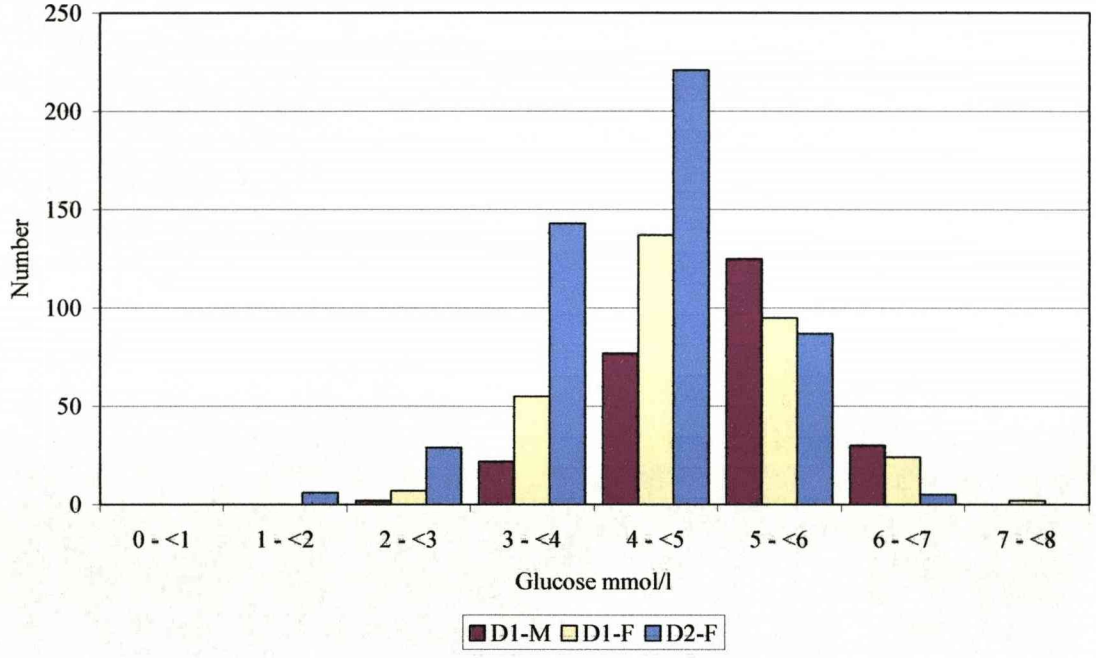

Figure 5.1 Distribution of average glucose concentrations in dataset D1-M, D1-F and D2-F.

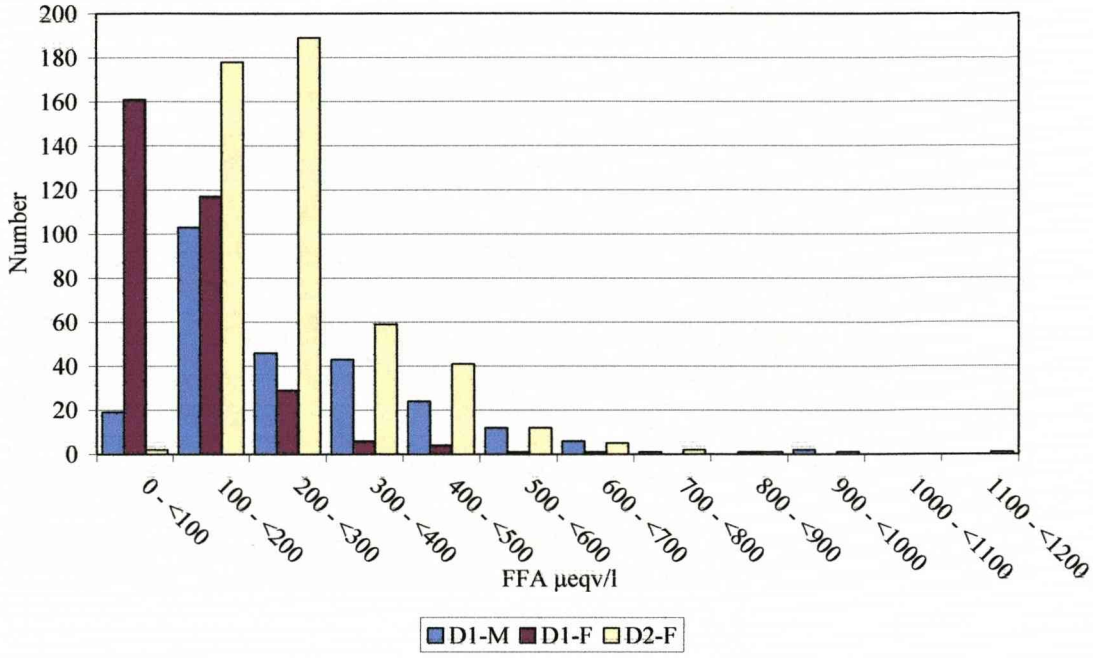

Figure 5.2 Distribution of average free fatty acids (FFA) concentrations in dataset D1-M, D1-F and D2-F.

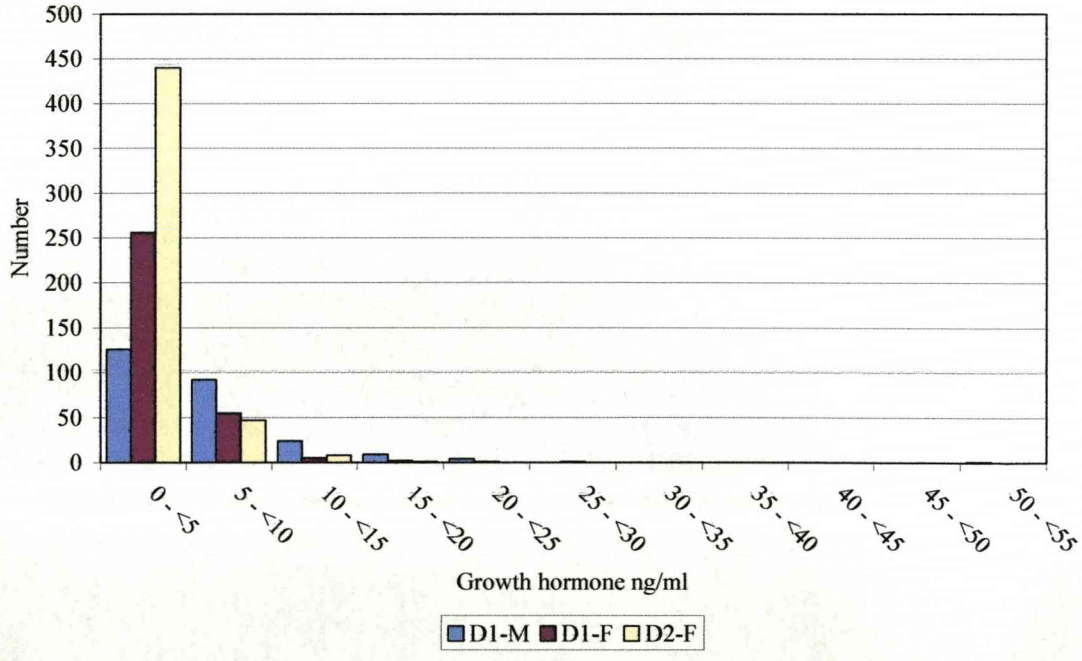

Figure 5.3 Distribution of average growth hormone concentrations in dataset D1-M, D1-F and D2-F.

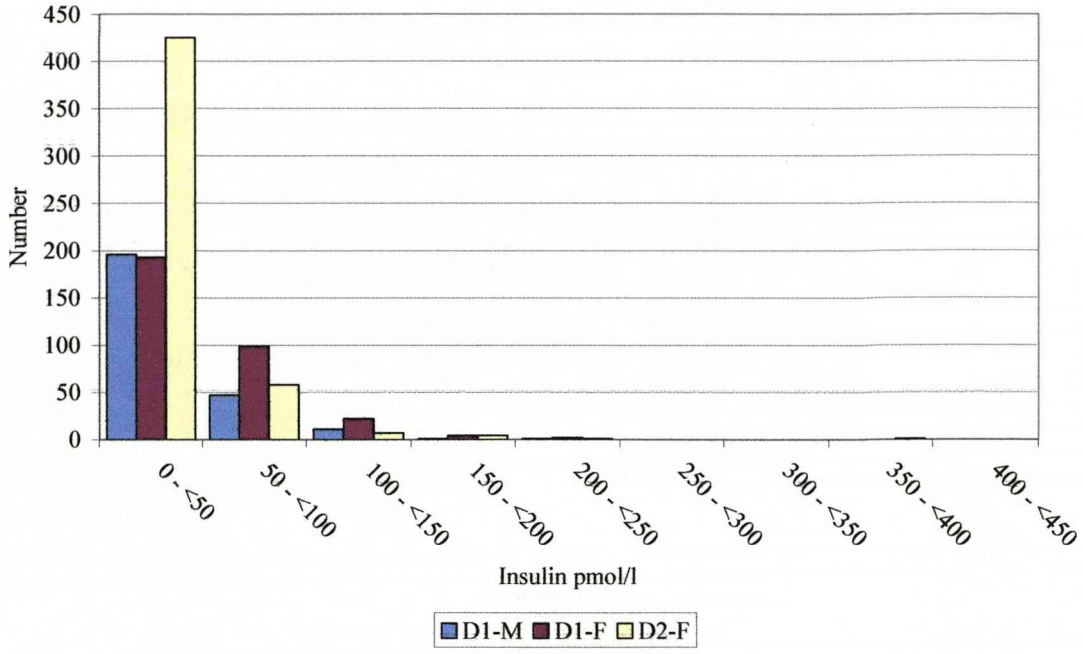

Figure 5.4 Distribution of average insulin concentrations in dataset D1-M, D1-F and D2-F.

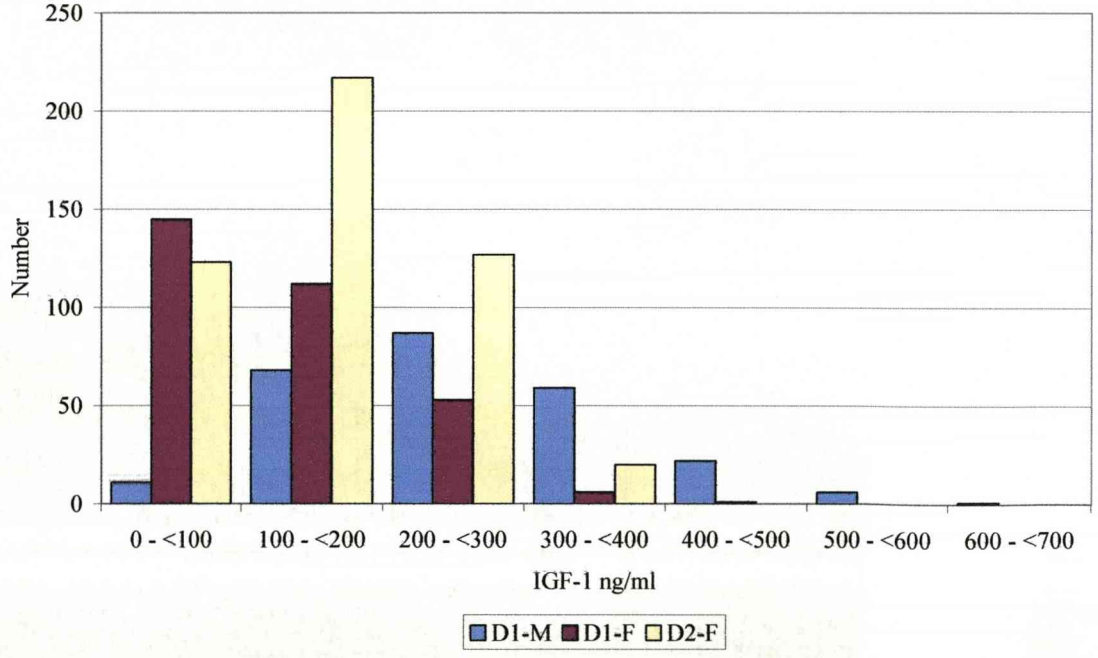

Figure 5.5 Distribution of average insulin like growth factor 1 (IGF-1) concentrations in dataset D1-M, D1-F and D2-F.

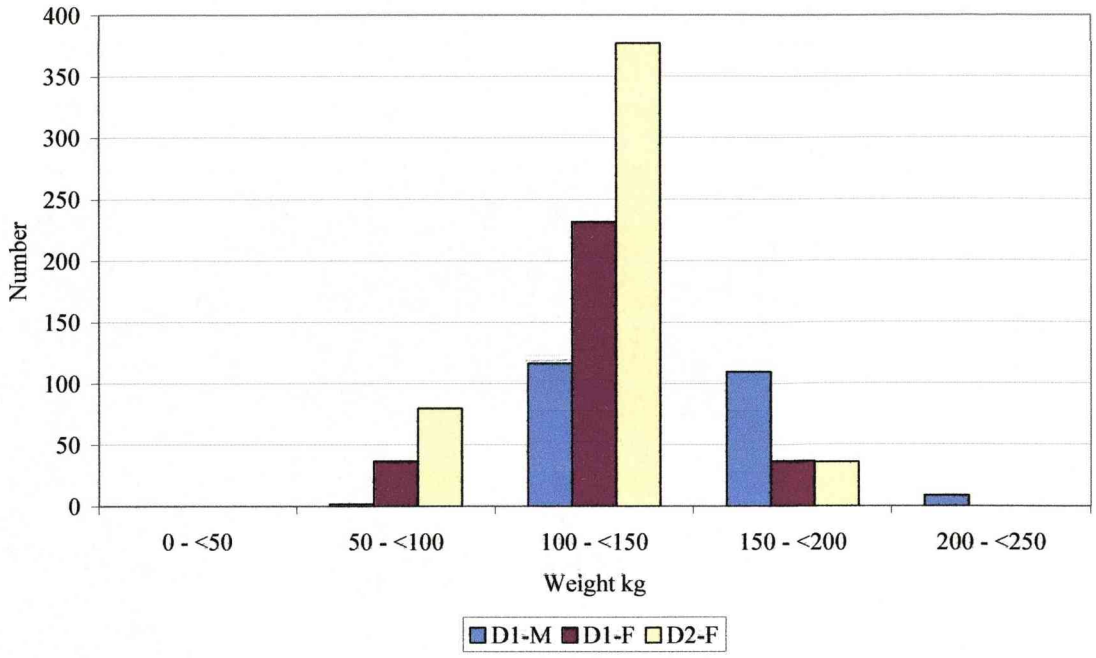

Figure 5.6 Distribution of average weight of calves in dataset D1-M, D1-F and D2-F.

5.3.2 Fixed and random effects

All random effects fitted were significant with the exception of the extra residual term D1-M in the glucose analysis though for consistency this term remained in the model (*Table 5.5*).

Table 5.5 Significance of the random effects in the model with D (Section 5.2.2.3) being approximately chi-squared distributed with 1 degree of freedom (NS = non significant P>0.05)

Trait	Random terms	D	Significance
Glucose	D1-M	1.18	NS
	D1-F	31.97	P<0.0005
	Additive genetic	6.81	P<0.025
FFA	D1-M	45.67	P<0.0005
	D1-F	83.18	P<0.0005
	Additive genetic	5.02	P<0.025
GH	D1-M	10.19	P<0.005
	D2-F	3.89	P<0.05
	Additive genetic	8.00	P<0.005
Insulin	D1-F	23.82	P<0.0005
	D2-F	44.77	P<0.0005
	Additive genetic	6.26	P<0.025
IGF-I	D1-F	125.37	P<0.0005
	D2-F	4.48	P<0.05
	Additive genetic	29.00	P<0.0005

The significance of the fixed effects for each hormone or metabolite is shown in *Table 5.6*. The fixed effect of batch, sex and sex.batch interaction was significant (P<0.001) in the case of glucose, FFA, GH, insulin and IGF-1. However, the fixed regression of sire percentage Holstein and age were not significant in any analyses with the exception of the effect of age in insulin and IGF-1 (P<0.001).

Table 5.6 The F test for significance of fixed terms fitted in each analysis. The denominator degrees of freedom is assumed to be infinity due to the dataset being >1000 (NS = non significant P>0.05)

Trait	Fixed term	Numerator df	F	Significance
Glucose	Sire % Holstein	1	0.13	NS
	Age	1	0.28	NS
	Batch	130	7.30	P<0.001
	Sex	1	57.33	P<0.001
	Sex.Batch	21	3.97	P<0.001
FFA	Sire % Holstein	1	0.03	NS
	Age	1	2.67	NS
	Batch	130	11.38	P<0.001
	Sex	1	391.75	P<0.001
	Sex.Batch	21	8.11	P<0.001
GH	Sire % Holstein	1	0.12	NS
	Age	1	1.50	NS
	Batch	130	2.11	P<0.001
	Sex	1	70.20	P<0.001
	Sex.Batch	21	2.67	P<0.001
Insulin	Sire % Holstein	1	0.05	NS
	Age	1	5.29	P<0.001
	Batch	130	9.66	P<0.001
	Sex	1	30.47	P<0.001
	Sex.Batch	21	6.90	P<0.001
IGF-1	Sire % Holstein	1	0.68	NS
	Age	1	19.67	P<0.001
	Batch	130	5.92	P<0.001
	Sex	1	419.76	P<0.001
	Sex.Batch	21	5.20	P<0.001

5.3.3 Heritability

The heritability estimates for each hormone or metabolite in each dataset are shown in *Table 5.7*. Heritability estimates varied within trait between the three datasets due to differences in the error variance, whilst the genetic variance was assumed to be constant (see *Table 5.2*). The standard errors although small tend to increase with the size of the heritability estimate. Furthermore, all heritability estimates for each trait were significantly different from zero ($P<0.0005$ to $P<0.025$). Glucose, FFA, GH and insulin all had moderate and similar heritabilities ($h^2 \pm$ standard error, range 0.09 ± 0.05 to 0.25 ± 0.13; see *Table 5.7*) whereas the heritability of IGF-1 was high.

Table 5.7 Estimated heritability for plasma FFA, glucose, GH, insulin and IGF-1 plus standard errors (s.e.) and significance in each subset.

Trait	**Dataset**	**n**	**h^2**	**s.e. (h^2)**	**Significance**
Glucose	D1-M	238	0.20	0.10	P<0.025
	D1-F	301	0.13	0.06	P<0.025
	D2-F	490	0.23	0.11	P<0.025
FFA	D1-M	238	0.12	0.06	P<0.025
	D1-F	301	0.09	0.05	P<0.025
	D2-F	490	0.25	0.13	P<0.025
GH	D1-M	238	0.13	0.06	P<0.005
	D1-F	301	0.18	0.09	P<0.005
	D2-F	495	0.15	0.07	P<0.005
Insulin	D1-M	238	0.22	0.12	P<0.025
	D1-F	301	0.12	0.06	P<0.025
	D2-F	495	0.10	0.06	P<0.025
IGF-1	D1-M	236	0.66	0.14	P<0.0005
	D1-F	298	0.21	0.05	P<0.0005
	D2-F	486	0.55	0.13	P<0.0005

5.3.4 Genetic and phenotypic correlations between hormone and metabolite data with weight

Table 5.8 shows the genetic and phenotypic correlations (± standard error) and heritability estimates for the six parameters (glucose, FFA, GH, insulin, IGF-1 and weight). Each bivariate analysis gave three estimates (D1-M, D1-F and D2-F) for the heritability and genetic correlations; though only the range is displayed (lowest to highest).

Table 5.8 Genetic correlations (above the diagonal), phenotypic correlations (below the diagonal, range from the lowest to highest) and heritability (on the diagonal, range from the lowest to highest) plus standard errors. (Significance only indicated for genetic correlation, NS = non significant P>0.05)

Trait	Glucose	FFA	GH	Insulin	IGF-1	Wt
Glucose	**0.09 ± 0.05 to 0.26 ± 0.12**	-0.18 ±0.39 NS	-0.46 ± 0.30 NS	0.67 ±0.30 NS	0.49 ± 0.24 NS	0.77 ± 0.23 P<0.025
FFA	0.20 ± 0.03 to 0.35 ± 0.04	**0.06 ± 0.03 to 0.28 ± 0.14**	-0.20 ± 0.35 NS	-0.24 ± 0.39 NS	0.14 ± 0.27 NS	0.26 ± 0.34 NS
GH	-0.07 ± 0.03 to -0.08 ± 0.04	0.00 ± 0.03 to 0.00 ± 0.04	**0.11 ± 0.06 to 0.19 ± 0.09**	0.39 ± 0.40 NS	-0.18 ± 0.26 NS	0.43 ± 0.31 NS
Insulin	0.25 ± 0.03 to 0.46 ± 0.04	-0.07 ± 0.02 to -0.16 ± 0.04	-0.07 ± 0.03 to -0.09 ± 0.04	**0.08 ± 0.05 to 0.23 ± 0.09**	0.71 ± 0.23 P<0.05	0.42 ± 0.28 NS
IGF-1	0.49 ± 0.03 to 0.62 ± 0.03	0.18 ± 0.03 to 0.24 ± 0.05	-0.04 ± 0.02 to -0.09 ± 0.04	0.27 ± 0.03 to 0.50 ± 0.04	**0.06 ± 0.02 to 0.65 ± 0.14**	0.47 ± 0.19 P=0.06
Wt	0.20 ± 0.03 to 0.28 ± 0.04	0.11 ± 0.03 to 0.18 ± 0.04	-0.02 ± 0.02 to -0.04 ± 0.04	0.14 ± 0.04 to 0.25 ± 0.04	0.19 ± 0.03 to 0.60 ± 0.04	**0.18 ± 0.07 to 0.31 ± 0.11**

The heritabilities produced by each bivariate analysis are comparable to those estimated by the univariate analysis, however, the range in heritabilities is large. The analyses to create *Table 5.8* produced 45 heritability estimates for each trait (15 bivariate analyses were done and estimates produced for each of the three datasets).

The genetic correlations of weight with glucose, FFA, GH, insulin and IGF-1 were positive and moderate in size (genetic correlation ± standard error, range 0.26 ± 0.34 to 0.77 ± 0.23). However, only the genetic correlations between weight with glucose and IGF-1 were significant. *Figure 5.7* illustrates the nature of the genetic correlations between the hormones and metabolites. All genetic correlations, illustrated in *Figure 5.7*, were non-significant with the exception of IGF-1 and insulin ($P<0.05$). The genetic correlation between FFA and glucose was negative as was the genetic correlation of FFA with insulin. This indicates that high FFA is associated genetically with low glucose and insulin concentrations. Furthermore insulin and glucose showed a moderate positive genetic correlation as did insulin and GH. Similarly insulin and glucose were both positively genetically correlated to IGF-1. Growth hormone showed a negative genetic correlation with IGF-1, glucose and FFA suggesting an inverse relationship between GH and the three parameters (IGF-1, glucose and FFA). Free fatty acids were positively genetically correlated with IGF-1. All the genetic correlations were moderate to large in size though the standard errors were also moderate to large this is probably due to the small size of the overall dataset used (n = 1077 total).

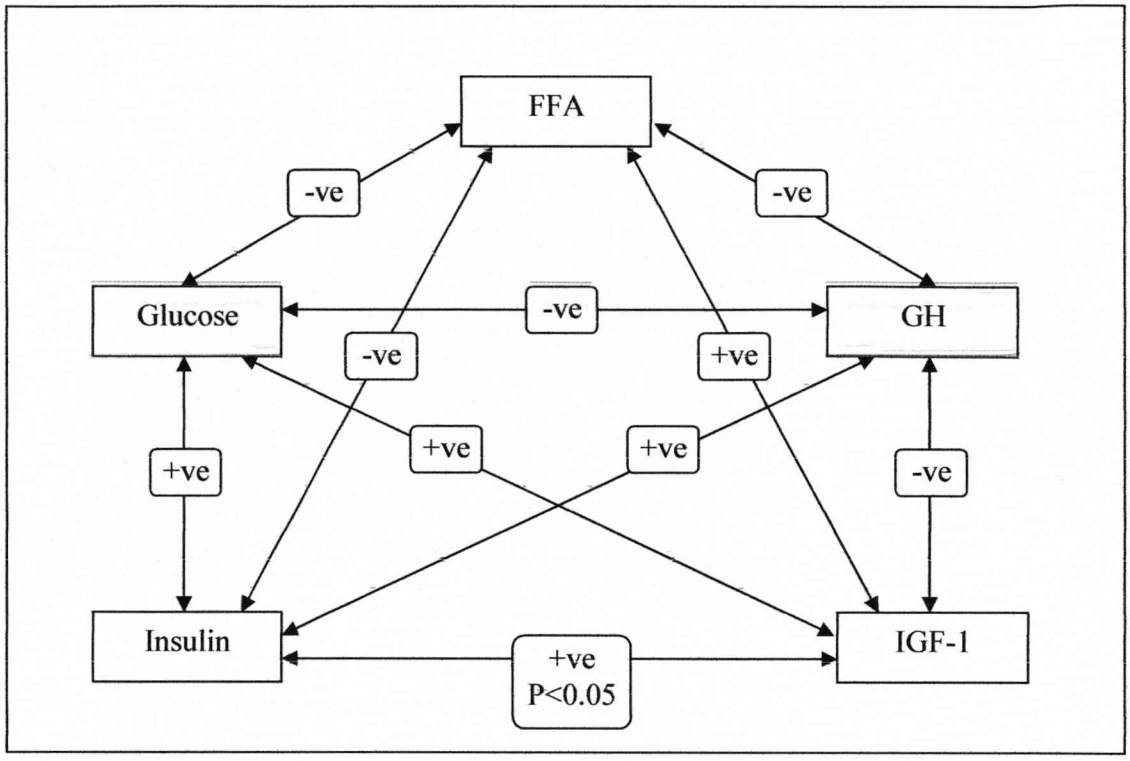

Figure 5.7 Illustration of the positive (+ve) and negative (-ve) genetic correlations between glucose, FFA, GH, insulin and IGF-1. Significance indicated if present.

Figure 5.8 illustrates the nature of the phenotypic correlations between the hormones and metabolites. The phenotypic correlations of weight with glucose, FFA, insulin and IGF-1 were positive and moderate with the exception of the correlation with GH which was negative although close to zero. The phenotypic correlation of IGF-1 with glucose, FFA and insulin were moderate and positive. Similarly the phenotypic correlation between insulin and glucose and FFA was positive and moderate in size. The phenotypic correlations of GH with glucose, FFA, insulin and IGF-1 were all close to zero. FFA and insulin showed a negative correlation which ranged from close to zero to moderate.

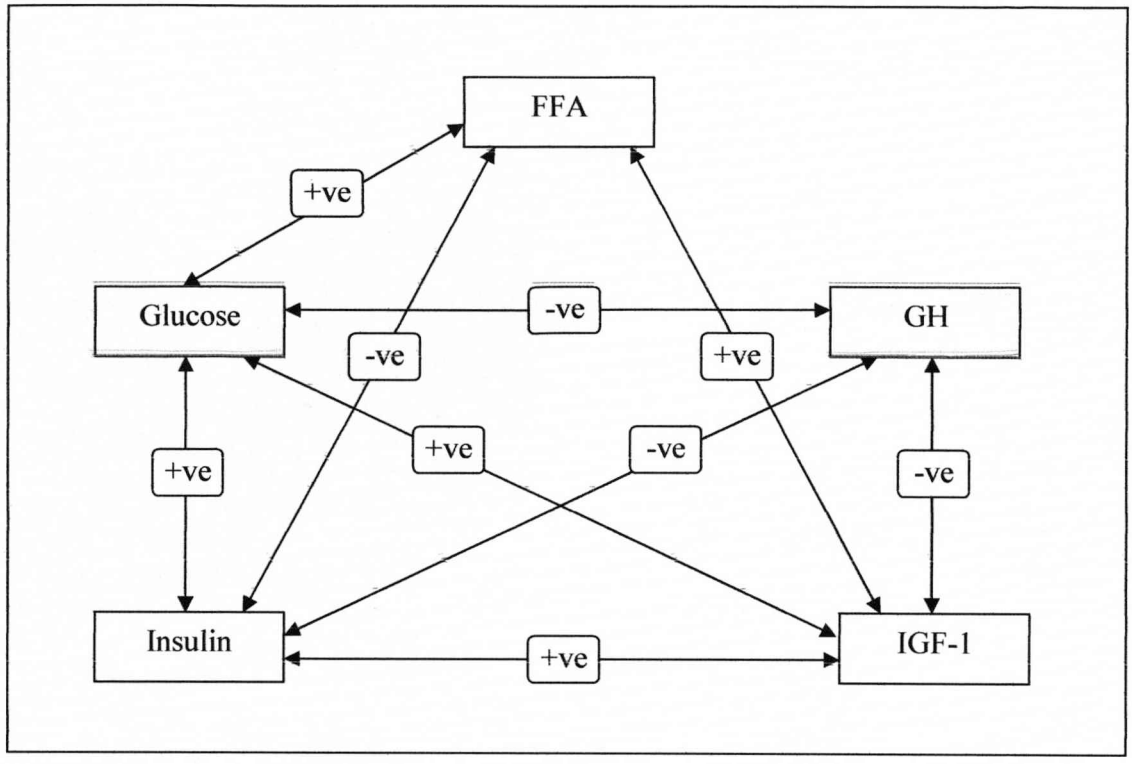

Figure 5.8 Illustration of the positive (+ve) and negative (-ve) phenotypic correlations between glucose, FFA, GH, insulin and IGF-1.

5.4 DISCUSSION

This study has found that additive genetic variation is responsible for a proportion of the phenotypic variation in glucose, FFA, GH, insulin and IGF-1 concentrations in four month old male and female Holstein-Friesian calves; all five having moderate and significantly different from zero heritabilities. Within trait, the heritability estimates differed for each dataset due to varied residual variance whilst the genetic variance remained consistent. The genetic correlations of IGF-1 with weight and insulin were significant and moderate. Furthermore, both genetic and phenotypic correlations between glucose, FFA, GH, insulin and IGF-1 concentrations with weight and each other were largely moderate with only few exceptions although not significantly different from zero.

5.4.1 Heritability of hormones and metabolites

This study has shown that significant ($P<0.0005$ to $P<0.025$) additive genetic variance is present in concentrations of FFA, glucose, GH, insulin and IGF-1 in male and female UK Holstein-Friesian calves (n = 1077, average age ± standard deviation, 126 ± 12.7 d). Furthermore, heritability estimates for these traits were moderate and significant (h^2 ± standard error, range 0.09 ± 0.05 to 0.66 ± 0.14; see *Table 5.7*). Within trait the estimates varied according to dataset, however this was due to inconsistencies in the residual variance, as described in detail in section 5.2.2.1.

The heritability of FFA was moderate in D1-M, D1-F & D2-F (h^2 ± standard error; 0.12 ± 0.06, 0.09 ± 0.05, 0.25 ± 0.13; $P<0.025$ respectively). Believing that the heritability estimate for D2-F is the most accurate because this dataset had the lowest amount of residual variance is incorrect because it assumes that the lowest amount of residual variance is closest to the actual residual variance due to error when all other factors e.g. sex, batch have been accounted for. However, this may indicate that the most accurate heritability estimate (i.e. data with the smallest amount of residual variance), with the exception of GH and insulin, is achieved in the dataset with the most tightly controlled experimental conditions of the three datasets, D2-F, and furthermore that a single hormone measurement for an animal can only be trusted when collected under such conditions. With the overall aim of this work being to identify juvenile predictors that can be easily used by the dairy industry this theory suggests that the measurement of the concentrations of these hormones can only be used under tight experimental conditions which are less likely to be achievable in farms or breeding companies.

For the purposes of interpretation of these analyses it is better to suppose that the true heritability for FFA, and indeed the other hormones and metabolites, lies within a range in this case of 0.09 ± 0.05 to 0.25 ± 0.13. This theory holds true in fact for all genetic studies in that the heritabilities estimated are specific for that dataset and depend largely on the amount of residual variance present. With large datasets (n = >5000) the confidence in the genetic parameter estimates increase because standard errors fall and

significance levels improve. Nevertheless it is only with repeated estimates in other datasets with agreeing results that the true heritability becomes clearer.

The estimates found in this study for FFA are similar to those found in Danish calves (Chapter 4) however lower than those estimated in a Danish study (Løvendahl and Jensen, 1997). That study estimated the heritabilities of plasma metabolites in male and female calves (Red Dane, Danish Friesian, Danish Jersey and Danish Red and White) following 24 hour fasting at 9 months of age and reported FFA to have higher heritability than found in the present data (males n = 198, $h^2 = 0.52 \pm 0.16$; females n = 190, $h^2 = 0.32 \pm 0.14$; Løvendahl and Jensen, 1997). The higher estimate for FFA heritability in the Løvendahl and Jensen (1997) study may be due to the low number of calves.

The heritability of glucose concentration was moderate in D1-M, D1-F & D2-F ($h^2 \pm$ standard error; 0.20 ± 0.10; 0.13 ± 0.06; 0.23 ± 0.11; $P<0.025$ respectively). These estimates are comparable to those in Danish calves (males, n = 1438, 269 ± 11d; $h^2 \pm$ standard error; 0.27 ± 0.06 see Chapter 4) and indeed comparable to estimates reported by Løvendahl and Jensen (1997) in Danish calves (males n=451, $h^2 = 0.22 \pm 0.08$; females n=371, $h^2 = 0.28 \pm 0.09$; Løvendahl and Jensen, 1997). A much higher heritability estimate was reported for glucose by Rowlands *et al.* (1983) which examined the genetic variation in glucose and other blood metabolites in fed young British Friesian bulls (n = 428; 3-15 months of age; glucose $h^2 = 0.41 \pm 0.17$). Though the Rowlands *et al.* (1983) estimate is from a smaller dataset and the range in ages is large and although this has been accounted for in the analysis it is not ideal.

Insulin concentration showed moderate heritability in D1-M, D1-F & D2-F ($h^2 \pm$ standard error; 0.22 ± 0.12; 0.12 ± 0.06; 0.10 ± 0.06; $P<0.025$ respectively). Furthermore these results were comparable to those found in older Danish male calves (Chapter 4; $h^2 \pm$ standard error; 0.21 ± 0.06). However insulin showed no genetic variation in male calves in a previous study by Løvendahl and Jensen (1997) (males n = 334, $h^2 = 0.04 \pm 0.08$) whilst the heritability of insulin in the female calves was much

higher (females n = 300, $h^2 = 0.43 \pm 0.11$). These results suggest that insulin in females is under different genetic control at 9 months than at 4-5 months of age. This is supported by the effect of age being significant only in the case of insulin and IGF-1 (discussed in section 5.4.2) indicating that over the range of ages at testing (range, 72 – 167 d) significant variation in the concentration of insulin and IGF-1 is due to age.

Growth hormone concentrations had moderate heritability in the present study ($h^2 \pm$ standard error; D1-M 0.13 ± 0.06; D1-F 0.18 ± 0.09; D2-F 0.15 ± 0.07; $P<0.005$). Baseline concentrations of GH are difficult to assess due to the pulsatility of secretion and often GH secretion is assessed following stimulation to overcome the random pulsatility. This method was used by Løvendahl *et al.* (1994) and Grochowska *et al.* (2001) who both calculated higher heritability estimates for stimulated GH (average heritability of the two studies 0.38) rather than for baseline concentrations (average heritability of the two studies 0.22).

Insulin like growth factor 1 in this study has shown high and significant heritability estimates particularly in the D1-M and D2-F datasets ($h^2 \pm$ standard error; D1-M 0.66 ± 0.14; D1-F 0.21 ± 0.05; D2-F 0.55 ± 0.13; $P<0.0005$). The estimates for D1-M and D2-F are higher than reported by Grochowska *et al.* (2001) in Polish Friesian male and female calves (n = 214, age 335 ± 8 d, $h^2 \pm$ standard error, 0.35 ± 0.14) however these calves are older and fewer in number than in the present study. Davis and Simmen (2000) obtained high estimates for IGF-1 heritability in a study using Angus beef cattle (n = 1220) divergently selected for IGF-1 concentrations in the post-weaning period (7 - 9 months of age). In this study IGF-1 concentrations at approximately 8, 8 ½ and 9 months of age showed high heritabilities ($h^2 \pm$ standard error; 0.32 ± 0.09; 0.59 ± 0.11; 0.31 ± 0.08 respectively) which are comparable to those in the present study.

Each hormone and metabolite analysed has shown moderate heritability. Furthermore these estimates, for the most part, are comparable to those found in previous studies in older calves of different breeds. This is encouraging particularly as different selection pressures are applied to different breeds (in this case Angus beef cattle, UK and Danish

Holsteins-Friesian, Polish Friesian, British Friesian, Red Dane, Danish Friesian, Danish Jersey and Danish Red and White). Although selection has not been specifically for FFA, glucose, GH, insulin and IGF-1 in the cattle populations discussed, with the exception of Davis and Simmen (2000; Angus beef cattle divergently selected for IGF-1), selection may have inadvertently caused changes in the inherent ability of a breed to produce FFA, glucose, GH, insulin and IGF-1. It is possible that concentrations of FFA, glucose, GH, insulin and IGF-1 will have been altered in different breeds due to selection for milk yield which has been applied in all dairy breeds to differing extents dependent on breed and country (Miglior *et al.*, 2005) and selection for growth and production traits applied in beef breeds (Simm, 2000b).

Concentrations of FFA, glucose, GH, insulin, IGF-1 and other hormones have been found to be altered in high and low genetic merit for milk yield lines. Basal and peak stimulated growth hormone levels have been shown to be significantly greater in high genetic merit for milk yield lines than in low lines (Løvendahl *et al.,* 1991; Beerepoot *et al.*, 1991; Woolliams *et al.,* 1993). Similarly, glucose, GH and insulin concentrations were found to be significantly higher in high genetic merit animals following overnight fast when compared to low genetic merit animals (n = 13 male, n = 11 female, between 8 – 18 days of age, Xing *et al.,* 1988). However conflicting results were published by Xing *et al.* (1991) who found no significant differences in basal insulin, GH and FFA in high and low genetic merit selection lines (n = 12 female calves, 6 – 8 months of age). Furthermore, recent work by Wathes *et al.* (2007) indicated that in multiparous cows (n = 312 Holstein-Friesian) insulin and IGF-1 showed a significant negative phenotypic correlation with milk yield at week four of lactation, however, in the case of IGF-1 the correlation became positive at week seven of lactation. This is possibly due to cows beginning to recover from negative energy balance and the down-regulation of liver GH receptors diminishing by week seven post-partum (Wathes *et al.*, 2007).

Although it would appear that differing genetic merit for milk yield is associated with differing concentrations of metabolic hormones (reviewed by Woolliams & Løvendahl, 1991) it would seem that genetically (i.e. heritability) and phenotypically (i.e.

concentrations) these hormones and metabolites are comparable in different breeds, particularly Holstein-Friesian and Danish dairy breeds as have been studied in datasets 1, 2 and 3 (Chapter 4 and 5) and also in other breeds referred to in the literature comparisons.

5.4.2 Fixed and random effects

The fixed effects of batch, sex and sex by batch interaction were significant ($P<0.001$) in the case of glucose, FFA, GH, insulin and IGF-1 (*Table 5.6*). It was expected that the effect of batch would be significant because it was fitted to account for many sources of variation. Blood samples for animals in D1-M, D1-F & D2-F were collected on 131 occasions (131 batches) over a period of 9 years (1996-2005) therefore batch includes variation due to time of sampling (e.g. person, day, month, season and year of sampling). Furthermore, the batch effect also contained variation due to farm in D2-F as no two farms in D2-F were ever sampled on the same day and the sex by batch interaction accounted for variation due to farm in D1-M and D1-F as these were sampled on the same day i.e. same batch, but at separate farms for each sex: therefore the sex by batch effect distinguished the two. Consequently the batch and sex by batch interaction accounted for variation due to for example diet and management effects specific to each farm.

The hormone or metabolite concentration was significantly affected ($P<0.001$) by the sex of calf sampled; males tending to have increased concentrations (with the exception of insulin; *Table 5.6*). Previous studies examining hormone concentrations in calves have also reported higher concentrations in males than females of the same age; with differences, due to sex, apparent in GH (5, 8 and 11 months of age, Keller *et al.,* 1979; birth – one year of age, Govoni *et al.,* 2003) and IGF-I (Plouzek & Trenkle, 1991; 16 week – one year of age, Govoni *et al.,* 2003). Although there is evidence to suggest that concentrations of these hormones and metabolites are higher in male than in female calves this does not mean that genetically they are different traits. It may be that males mature at a faster rate and therefore concentrations are higher at a younger age than in

females. In the present study the genetic variation of each hormone and metabolite were similar in the three datasets (D1-M, D1-F & D2-F). Furthermore, the heritability estimates for the three datasets for each hormone or metabolite were similar (heritability range, 0.09 – 0.25), the exception being IGF-1 whose heritability estimates were higher and more varied (heritability range, 0.21 – 0.66).

The fixed regression of sire percentage Holstein was not significant in any analysis. The sire percentage Holstein was fitted to account for breed differences between the animals. Over the period of sampling (1996-2005) the percentage of Holstein genes in the animals will have altered and therefore fitting sire percentage Holstein attempts to account for these subtle differences. Other studies have reported the sire percentage Holstein to have a significant affect on reproduction and milk production data (e.g. Hoekstra *et al.*, 1994; Royal *et al.*, 2002). However in the present analysis it did not have a significant effect probably because the mean sire percentage Holstein was high 99.62 and the range was small (87.5 % to 100 %).

The fixed regression of age was not significant in any analysis with the exception of insulin and IGF-1. It may be possible that the concentrations of insulin and IGF-1 are more affected by age, particularly within the period of testing (range, 72 – 167 d). Concentrations of IGF-1 and insulin increase with age (birth until ≥15 months of age; Plouzek & Trenkle, 1991; Skaar *et al.*, 1994). In contrast, glucose (Rowlands *et al.*, 1983) and FFA (Quigley *et al.*, 1991) remain relatively stable over the range of ages at testing (range, 72 – 167 d) and vary due to feeding, whereas GH concentrations decrease with age (birth until ≥52 weeks of age; Govoni *et al.*, 2003). However, the release of GH is less stable than both IGF-1 and insulin in that large secretory pulses of GH add noise to the data and add to the total variance (Theilgaard *et al.*, 2007). Growth hormone concentrations for animals in D1-M, D1-F & D2-F were the mean of two plasma samples taken 15 or 30 minutes apart (See Chapter 3 for sampling details) and furthermore the data was not edited to remove random pulses of GH (mainly due to the small dataset, n = 1074) and therefore it is possible that the variance due to the

pulsatility of GH in part masked the effect of age and therefore age did not appear to be significant in the analysis.

5.4.3 Hormones and metabolites

The concentrations of glucose, FFA, GH, insulin and IGF-1 found in this study are comparable to those found in previous studies in calves of similar ages (Rowlands *et al.*, 1983; Wooliams *et al.*, 1992; Løvendahl *et al.*, 1994; Taylor *et al.*, 2004b; Klotz & Heitman, 2006). Furthermore the concentrations of glucose, GH and insulin found in this study are comparable to those found in older Danish male calves (D3; see Chapter 4). The average concentration of FFA found in this study is however lower than that found in Danish male calves (D3; see Chapter 4). This is most likely due to the calves in D3 being fasted prior to sampling unlike those in D1 & D2. Concentrations of FFA increase greatly during fasting due to mobilisation from adipose tissue thus the animals in D3 will have raised concentrations of FFA (Discussed in Chapter 4).

The mean concentration of each hormone or metabolite varies dependent on dataset. Concentrations were higher in male calves (D1-M) with the exception of insulin where female calves in D1-F were higher. However D1-F had a large amount of additional residual variance and little genetic variance when analysed separately thus lowering the certainty in the insulin concentrations of animals in D1-F. The irregularities in the residual variances particularly in D1-F and D1-M are likely to be due to inconsistencies in experimental procedure. The experimental protocol required animals to be transported to the experimental site (D1-M & D1-F) or grouped and penned (D2-F) 1-2 hours prior to sampling to allow acclimatization. This did occur in D2-F and less often in D1-M but infrequently in D1-F. On some occasions calves were transported to the experimental site immediately prior to sampling giving these animals minimal time to adjust to the experimental environment prior to sampling and possibly causing them increased stress.

Stress can be broadly divided into physical stress, e.g. feed deprivation, temperature, and psychological stress, e.g. interaction with humans, restraint, handling, and separation (Grandin, 1997). In the case of this study, any stress caused will be psychological due to transport to the experimental site, unfamiliar surroundings, and handling. Although the experimental protocol was designed to minimize the possible stressors by allowing time to acclimatize etc in some cases, as described above, the calves may not have been given sufficient time to recover from stress caused by transport and acclimatize to new surroundings. Stress can be determined and quantified, to a degree, by measuring circulating cortisol concentrations, beta endorphin concentrations and heart rate (Grandin, 1997). Increased concentrations of cortisol, released from the adrenal cortex in response to adrenocorticotrophic hormone (ACTH) from the anterior pituitary which in turn is stimulated by corticotrophic releasing hormone (CRH) from the hypothalamus, are seen during periods of stress (Evans *et al.*, 1993; Dorin *et al.*, 1996; Grandin, 1997). Under normal conditions concentrations of cortisol are regulated by negative feedback of cortisol on CRH (Evans *et al.*, 1993; Dorin *et al.*, 1996) however during periods of stress cortisol concentrations can rise greatly dependent on the severity of the stressors (Grandin, 1997). Furthermore, stress has been reported to increase concentrations of adrenaline, noradrenaline, glucose, FFA, and beta hydroxybutyrate (reviewed by Obernier & Baldwin, 2006).

The effects of stress on both glucose and FFA, due to increased gluconeogenesis and lipolysis, are particularly important in the interpretation of these results and indeed the anomalies in the variance components for each dataset. In the case of FFA concentration it is known that it is particularly affected by stress and the time since last feeding. A study by Fox *et al.* (1991) in Holstein-Friesian heifer calves (n = 4, three months of age) found FFA concentrations showed diurnal variation with effects seen due to time relative to last feed. Diurnal variations in FFA have also been seen in goats (Alila-Johansson *et al.*, 2004) and bovids (Zanzinger *et al.*, 1994). Diurnal variation in FFA is particularly a concern in datasets D1-M and D1-F as these animals were not fasted and although food was available during the sampling, it is likely that intake will be affected by the length of time left to acclimatize to the experimental conditions.

Furthermore, it is likely that feeding in some animals will be disrupted by the transportation, human interaction and the unusual environment (particularly in D1-M and D1-F). Fox *et al.* (1991) suggested that variation in FFA concentration in response to stress could be reduced by regular handling before sampling which was not possible in the present study. Literature regarding the affects of stress on concentration of GH, insulin and IGF-1 are limited. Cortisol has an antagonistic effect on insulin to allow gluconeogenesis (Lager, 1991) and therefore perhaps concentrations of insulin during periods of stress are unaltered. Growth hormone and IGF-1, in conjunction with 11beta-hydroxysteroid dehydrogenase, are thought to be involved in regulating the ratio of active cortisol and inactive cortisone (Stewart *et al.,* 2001). Growth hormone and IGF-1 appear to inhibit the conversion of inactive cortisone to active cortisol and thereby reduce cortisol concentrations (Stewart *et al.,* 2001) however whether concentrations of GH and IGF-1 are affected by cortisol is unclear.

In addition to diurnal variation in FFA (Fox *et al.*, 1991; Zanzinger *et al.*, 1994; Alila-Johansson *et al.*, 2004) concentrations of glucose, insulin and GH show diurnal variation. A study by Ndibualonji *et al.* (1997) highlighted this variation in plasma concentrations of metabolites in non-pregnant and non-lactating cows (n = 4, Friesian, 47.0 ± 6.3 months of age) fed diets varying in energy and nitrogen content for 172 days in total. Significant variation ($P<0.05$) was seen between different sampling time periods (postprandial, nocturnal inter-prandial, morning postprandial, diurnal inter-prandial and a non-fed period with samples taken every 10 minutes by jugular catheter) for insulin, glucose and GH even during a period of feed deprivation (16 hours; Ndibualonji *et al.,* 1997). Similarly diurnal variation in FFA, glucose, GH and insulin concentrations was shown in a study by Xing *et al.* (1991) in Friesian heifers (n = 12, 6-8 months of age) of high and low genetic merit line fed at 75% and 125% maintenance energy requirement. Diurnal variation and variation due to time elapsed since last feeding can not be accounted for in the genetic analysis of the present data (D1-M, D1-F & D2-F). This highlights two sources of variation which could partially account for the unequal variation in the three datasets, hence reduced heritability estimates for each hormone or metabolite.

5.4.4 Genetic and phenotypic correlations between hormone and metabolite data with weight

This study has found mainly non-significant, although moderate in size, genetic and phenotypic correlations between FFA, glucose, GH, insulin, IGF-1 and weight in male and female UK Holstein-Friesian calves (n = 1077, average age ± standard deviation, 126 ± 12.7 d). Of the 15 genetic correlations estimated 3 were significant between weight and glucose (genetic correlation ± standard error, 0.77 ± 0.23, P<0.025), weight and IGF-1 (0.47 ± 0.19, P=0.06) and between IGF-1 and insulin (0.71 ± 0.23, P<0.05; *Table 5.8*). The significance of the phenotypic correlations was not assessed directly, however, significance is implied when the correlation is greater in size than 2 x standard error. All phenotypic correlations were significant except the phenotypic correlation between weight and GH and between GH and FFA.

The genetic correlations of weight with glucose, FFA, GH, insulin and IGF-1 are positive and moderate (range genetic correlation ± standard error, 0.26 ± 0.34 to 0.77 ± 0.23) although only significant between weight and both glucose and IGF-1. The phenotypic correlations between weight with glucose, FFA, insulin and IGF-1 were positive, moderate and significant (range phenotypic correlation ± standard error, 0.20 ± 0.03 to 0.62 ± 0.03) with the exception of the phenotypic correlation between weight with GH (-0.07 ± 0.03 to -0.08 ± 0.04). This indicates that at both a genetic and phenotypic level the concentrations of the five hormones and metabolites increase in line with weight. Although not directly comparable, previous work has examined the correlations between hormones and metabolites with growth rate and body condition score. A study by Govoni *et al.* (2003) examined the changes in GH, IGF-1 and average daily gain (ADG) in Hereford calves (n = 8 male, n = 8 female) from birth until one year of age by weekly sampling. Treating the weekly samples as repeated measures (total n = 424) phenotypic correlations were calculated. In contrast to the findings made here with weight (*Table 5.8*) Govoni *et al.* (2003) reported that GH was negatively correlated to ADG (males -0.18, females -0.09). However IGF-1 was positively correlated to ADG (males 0.21, females 0.12; Govoni *et al.*, 2003). Further to this, Wathes *et al.* (2007)

reported IGF-1 to be positively phenotypically correlated to BCS in primiparous cows (Holstein Friesian n = 188) at weeks two and four and in multiparous cows (Holstein-Friesian n = 312) at weeks four and seven post-partum. This study also found BCS was positively correlated to insulin in multiparous cows at week 4 post-partum (Wathes *et al.*, 2007). Rowlands *et al.* (1983) found glucose to be positively genetically (genetic correlation ± standard error, 0.30 ± 0.38) and phenotypically correlated (0.07) to growth rate in young British Friesian bulls (n = 428; 3-15 months of age) and furthermore at 18-20 weeks of age glucose was phenotypically correlated to both growth rate and weight (0.26 and 0.07 respectively). This highlights that although comparisons can not be directly made between growth rate, BCS and weight, it is expected that these traits are genetically correlated and therefore the results in these studies described support the results in the present study with the exception of GH and ADG (Govoni *et al.*, 2003).

Both glucose and insulin showed a negative genetic correlation to FFA in the present study (genetic correlation ± standard error, -0.18 ± 0.39 and -0.24 ± 0.39 respectively). This is plausible because FFA increase due to lipolysis during periods of energy shortage and they are a good indicator of energy balance and concentrations of glucose and insulin are positively correlated to energy balance in the post-partum period (Reist *et al.*, 2002). These correlations are partly supported by work by Løvendahl and Jensen (1997) who reported a negative genetic correlation between FFA and insulin in male and female calves (genetic correlation ± standard error, -0.28 ± 0.41 and -0.73 ± 0.27 respectively), however, the genetic correlation of FFA with glucose was positive (0.32 ± 0.28 males and 0.24 ± 0.30 females). These calves had been fasted for 24 hours prior to sampling. It is possible that after several hours of fasting glucose concentrations rise in response to gluconeogenesis in the liver, however, since glucose is relatively stable in ruminants this rise possibly will only occur after fasting has first caused glucose concentration to fall which takes between 24-48 hours of fasting (Diskin *et al.*, 2003; Chelikani *et al.*, 2004). Wathes *et al.* (2007) also reported a negative phenotypic correlation between FFA and insulin at several time points post-partum in both primiparous (n = 188) and multiparous (n = 312) cows.

In the current study, insulin showed a positive genetic correlation with glucose, GH and IGF-1 (genetic correlation ± standard error, range 0.39 ± 0.40 to 0.71 ± 0.23) suggesting that at a genetic level, all three increase with increasing insulin. Insulin's primary function is glucose regulation and it is released, from the islets of Langerhans in the pancreas, in response to glucose concentration rising above a critical level (Jiang and Zhang, 2003). The link between insulin with GH and IGF-1 is less straightforward. IGF-1 is released in response to GH and concentrations of both IGF-1 and GH are controlled by feedback mechanisms to the hypothalamus and the anterior pituitary (See Chapter 1; Clark & Robinson, 1996). Furthermore IGF-1 was positively genetically correlated to glucose. Due to the positive genetic correlation between insulin and glucose it is expected, though not inevitable, that the correlation of them both with IGF-1 would be in the same direction, in this case positive.

FFA showed a positive weak genetic correlation with IGF-1 and a weak negative correlation to GH. GH and IGF-1 were weakly negatively genetically correlated. These three relationships imply that when FFA concentrations are high, IGF-1 will also be high yet GH will be reduced. Similar correlations were reported by Wathes *et al.* (2007) who found FFA was negatively correlated to IGF-1 in primiparous (n = 188) and multiparous (n = 312) cows at weeks -1, 2 and 4 postpartum (GH was not studied). It is likely that the cows in the Wathes *et al.* (2007) study were experiencing negative energy balance and therefore an energy shortage, however this does not seem to have affected the correlations when compared to fed calves in the present study. However, in the present study, animals sampled (D1-M, D1-F & D2-F) may have been experiencing the effects of stress (discussed section 5.4.3). This negative genetic correlation between IGF-1 and GH has also been reported by Grochowska *et al.* (2001) in Polish Friesian cattle (male and female n = 214, age 335 ± 8 d, -0.26 ± 1.21) however the standard error of this genetic correlation was high (1.21). Govoni *et al.* (2003) also reported a negative phenotypic correlation between IGF-1 and GH in Hereford calves (n = 8 male, n = 8 female; birth until one year of age weekly samples). In contrast to our findings, Løvendahl and Jensen (1997) found GH and FFA were positively genetically correlated in calves (genetic correlation ± standard error, males 0.40 ± 0.33; females 0.16 ± 0.30).

These animals had been fasted for 24 hours before sampling and therefore FFA concentrations were high (FFA males 716 μeqv/l, females 609 μeqv/l), in comparison to the animals in the present study (FFA range 126.77 to 261.97 μeqv/l; *Table 5.4*). Furthermore, GH concentrations are raised during periods of energy shortage (Chelikani *et al.,* 2004; Diskin *et al.,* 2003). This suggests that GH and FFA are negatively correlated during normal feeding and positively correlated during periods of energy shortage such as a 24 hour fast (Løvendahl and Jensen, 1997) which could be due to different genes being switched on during different energy states.

Glucose and GH were negatively genetically correlated (genetic correlation ± standard error, -0.46 ± 0.30) in the present study, however, a positive correlation was reported by Løvendahl and Jensen (1997) which again highlights the importance of energy status when measuring hormone concentrations and correlations. During normal feed allowance it is logical that glucose will be low and yet GH high such as during the night. Conversely after 24 hours of fasting GH concentrations will be high and glucose concentrations may also have increased due to gluconeogenesis in the liver occurring.

Caution should be used when interpreting the genetic and phenotypic correlations involving GH because it shows random pulsatility which can add to the total variance if data is not first inspected to remove measurements that appear to be peak rather than basal concentrations (Theilgaard *et al.,* 2007). GH data was not edited before use because of the small size (n = 1074) of the dataset, the possible effects of stress and feed intake making it unclear at what point to distinguish between basal rather than peak GH.

Phenotypic correlations found in this study were, in the most part, significant and in the same direction as the genetic correlations and exceptions were close to zero.

5.4.5 Future work

This research has indicated that an appropriate level of genetic variation is present in all traits investigated to potentially be useful for juvenile selection criterion provided this is

following a strict experimental procedure for some hours before sampling and management practices within the population of animals used is stringent to avoid additional error variance. As discussed previously, with all genetic studies when analyses are repeated in further datasets the confidence in the genetic parameter estimates increases. It would be beneficial to do similar analyses on these metabolites and hormones in data containing more male calves and perhaps with less apparent environmental variance.

These analyses have shown that genetic and phenotypic relationships are present between FFA, glucose, GH, insulin, IGF-1 and weight. Genetic correlations were mainly not significant however this is probably due to the small size of the dataset. The genetic and phenotypic correlations do suggest that selection for one of the traits (FFA, glucose, GH, insulin, IGF-1) is likely to change the others and weight at a genetic level. Additionally the findings of previous studies (see Section 5.4.1) detailing differences in these hormones and metabolites in high and low genetic merit for milk yield selection lines would suggest that selection for one of these may cause changes in milk yield.

With the data that are currently available (D1-M, D1-F and D2-F) the next stage of this work will be to investigate the genetic and phenotypic (co) variation between these traits and fertility and other traits of economic importance such as milk, protein and fat yield (Chapter 6).

Chapter 6: GENETIC AND PHENOTYPIC CORRELATIONS BETWEEN METABOLITES AND HORMONES IN UK HOLSTEIN-FRIESIAN CALVES WITH TRAITS OF COMMERCIAL IMPORTANCE INCLUDING FEMALE FERTILITY

6.1 INTRODUCTION

Selection for female fertility is limited by three major constraints; fertility traits can only be assessed in the female, after first calving at approximately 2 years of age and traditional parameters have low heritability (discussed in Chapter 1). It could be possible to overcome some of these limitations by finding a prepubertal indicator trait for female fertility. This would possibly allow selection decisions for female fertility to be made sooner on both males and females, thus reducing the long generation interval and with more certainty due to the moderate heritability and genetic correlation to female fertility (Chapter 1).

In order to be an effective juvenile indicator trait, in addition to having a moderate heritability (Chapter 5), the trait must be genetically correlated to the breeding goal in question, in this case female fertility. Furthermore, if the trait is unfavourably genetically correlated to traits of commercial importance such as production, care must be taken when selecting for the indicator trait to prevent detrimental side effects to other traits. Often important selection criteria are unfavourably genetically correlated with other traits of importance, for example it is widely understood that milk yield is negatively genetically correlated to female fertility (e.g. Hoekstra *et al.*, 1994; Pryce *et al.*, 1997; Royal *et al.*, 2002; Veerkamp *et al.*, 2000). Provided that the genetic correlation between the two traits is not 1 or -1, then with careful consideration, selection indices can be designed with relative weightings on several parameters to allow improvements to be made in a number of traits simultaneously. Despite the unfavourable genetic correlation between fertility and milk yield many countries select for female fertility through fertility indices. Predicted transmitting abilities (PTA) for

several fertility traits are weighted according to relative economic value and combined in an index. This has been done recently to develop the UK fertility index which combines PTAs for calving interval and non return rate 56 days after insemination (NR56; See Chapter 1; Wall *et al.*, 2003a). To prevent unfavourable response to selection it is important that care is taken when developing breeding programs for individual farms and the industry as a whole. If strong selection pressure is placed on few traits although the traits of interest are likely to improve, traits unfavourably correlated to these may deteriorate e.g. somatic cell count or conception rate to first service. Unfavourable correlated response to selection can be managed through careful consideration of selection goals and by not focusing strongly on few traits of interest.

Following on from Chapter 5, which found that an appropriate amount of genetic variation is present in free fatty acids (FFA), glucose, growth hormone (GH), insulin and insulin like growth factor 1 (IGF-1) in dairy calves to be potentially used as juvenile selection criterion, the aim of this chapter was to estimate the genetic and phenotypic (co) variation of these hormones and metabolites with fertility and production. Physiological fertility parameters based on milk progesterone measurement and sire PTAs for milk production and female fertility will be analysed.

6.2 MATERIALS AND METHODS

6.2.1 Animals and sampling

This analysis, like Chapter 5, uses Dataset 1 (D1) and 2 (D2; described in detail in Chapter 3). Plasma samples were collected from Holstein-Friesian calves (average age days ± standard deviation; 126 ± 12.7 d) during two studies forming two datasets; Dataset 1 (D1-F, n = 326 females, D1-M, n = 256 males; 2002-2006; MOET breeding scheme) and Dataset 2 (D2-F, n = 496 females; 1996-2001; 7 commercial dairy herds; see Chapter 5; *Table 5.1*). Plasma samples were analysed for FFA, glucose, GH, insulin and total IGF-I (see Chapter 2 for assay details).

6.2.2 Physiological fertility parameters

Milk progesterone data collected during the studies and included in datasets D1-F and D2-F were used to calculate a number of physiological fertility parameters (described in Chapter 3). The interval to commencement of luteal activity postpartum (CLA; characterised as the interval from calving until milk progesterone concentration is ≥ 3ng/ml for at least 2 consecutive samples when sampling is carried out 3 times per week; Royal *et al.*, 2000b), was calculated for 202 of the females in D1-F and for 232 of the females in D2-F (*Table 6.1*).

Furthermore, a fertility measure introduced by Petersson *et al.* (2006a; 2006b) was used. This was calculated as the percentage of milk samples with luteal activity (milk progesterone ≥ 3ng/ml) within 60 days postpartum (PLA). PLA was calculated using all milk samples (three times per week) within 60 days postpartum (PLA_a, n = 343), using the first milk progesterone sample per week (PLA_w, n = 343), the first milk progesterone sample per fortnight (PLA_f, n = 343) and a randomly selected sample during the first four weeks and thereafter taken at monthly intervals (PLA_m, n = 296; *Table 6.1*; Described in Chapter 3).

6.2.3 Sire predicted transmitting abilities (PTA)

For 1035 of the 1078 animals in D1-M, D1-F and D2-F combined, sire PTAs (from sire progeny test for 140 sires; MDC Breeding, Milk Development Council, Stroud Road, Cirencester, Gloucestershire and calculated by Edinburgh Genetic Evaluation Service; SAC, Bush Estate, Penicuik) were available. Although estimation of genetic correlations between these and the hormone or metabolite data was not possible, genetic regressions of the hormone or metabolite data on the sire PTAs was carried out. Therefore the sire PTA was fitted in the univariate model as a covariate effect. Significance levels of these were obtained and indicative of a potential genetic relationship between the two.

Sire evaluations in UK dairy cattle contain many different PTAs for production, conformation, fertility and health traits. In addition to this, several indices are presented for example PIN, PLI (See Chapter 1). Sire PTAs (for 140 sires) were obtained (August and November 2006) and those fitted separately as covariates were:

305d milk yield kg

305d fat yield kg

305d protein yield kg

Fat percentage

Protein percentage

Production index (PIN) - based on the PTAs for kg milk, fat and protein,

Profitable lifetime index (PLI) - based on the PTAs for kg milk, fat, protein, lifespan, somatic cell count (SCC), locomotion and fertility

Fertility index - based on calving interval and non return rate 56d after insemination

Non return rate 56 days after insemination (NR56)

Calving interval

DIM first AI – Days in milk until the first AI

Number of services per conception

Condition score – Ranging from 1 (thin) to 9 (fat)

6.2.4 Statistical analysis

6.2.4.1 Physiological fertility parameters

The CLA data was $\log_{10}$ transformed to give normally distributed residuals (*Figure 6.1*). Although PLA_a, PLA_w, PLA_f and PLA_m (coefficients of skewness -0.155; 0.041; 0.290; 0.318 respectively) were not exactly normally distributed and had some anomalous results, $\log_{10}(x)$, square root(x), 1/(x) or $(x)^2$ transformations did not improve (bring closer to zero) the coefficients of skewness and therefore the data was not transformed (*Figures 6.2 – 6.5*). Fertility data (CLA, PLA_a, PLA_w, PLA_f and PLA_m) from Dataset 1 and Dataset 2 (D1-F and D2-F) were analysed separately first to assess the possibility of

combining them. The residual and genetic variance present in each dataset was compared (*Table 6.1*). Univariate mixed models were fitted to the fertility data using ASREML software (Gilmour *et al.,* 2006). The inclusion of various components in the model was explored. The fixed effects in the initial model were sire percentage Holstein, year of calving, season of calving and the farm. The genetic relationships were modeled by the relationship matrix calculated from the three-generation pedigree (over 4600 animals). The initial univariate model fitted to the fertility data for each subset was:

$$Y_{ijkl} = \alpha + {}_{b1}P + Y_i + S_j + F_k + A_l + \varepsilon_{ijkl}$$

Where:

Y_{ijkl} = the physiological fertility parameter

the fixed effects are:

α = intercept

${}_{b1}P$ = regression variable of sire percentage Holstein with coefficient *b1*

Y_i = the effect of year of calving (i = 1 to 8)

S_j = the effect of season of calving (j = 1 to 4)

F_k = the effect of farm (k = 1 to 8)

and the random effects are:

A_l = breeding value ($N(0, \sigma_A^2\mathbf{A})$ where A is the numerator relationship matrix of animals available in the data)

ε_{ijkl} = error term ($N(0, \sigma_E^2)$)

From this analysis the genetic and residual variance (± standard error) for each subset (D1-F and D2-F) and for each physiological fertility parameter was obtained (*Table 6.1*).

Table 6.1 Mean (before fitting the model), interval to commencement of luteal activity postpartum (logCLA; log_{10} units), percentage of samples with luteal activity within 60 days postpartum using all (PLA_a), weekly (PLA_w), fortnightly (PLA_f) and monthly milk samples (PLA_m), genetic variance (± s.e.) and the residual variance (± s.e.) for each subset (D1-F and D2-F).

Trait	Dataset	Mean	σ^2_A ± s.e.	σ^2_E ± s.e.
logCLA	D1-F	3.50	0.04 ± 0.04	0.20 ± 0.04
	D2-F	3.16	0.15 ± 0.07	0.13 ± 0.06
PLA_a	D1-F	39.12	56.54 ± 77.46	471.12 ± 81.37
	D2-F	51.70	312.30 ± 216.88	179.86 ± 179.86
PLA_w	D1-F	35.71	19.60 ± 65.32	519.93 ± 78.65
	D2-F	48.15	401.38 ± 271.20	175.93 ± 222.69
PLA_f	D1-F	32.74	38.82 ± 82.59	612.71 ± 94.55
	D2-F	42.44	215.16 ± 219.55	319.20 ± 192.29
PLA_m	D1-F	34.83	0.00 ± 0.00	1056.01 ± 115.16
	D2-F	45.76	567.15 ± 616.46	730.84 ± 537.38

Within the physiological fertility parameters (CLA, PLA_a, PLA_w, PLA_f and PLA_m) the genetic variance and residual variance for each of the two subsets varied and they had large standard errors. This is probably due to the two subsets (D1-F and D2-F) being small in size. The size of the residual variance in D2-F was higher all cases. It was decided because of the small subset size and the large differences in residual and to a lesser extent genetic variance that the inclusion of extra random terms fitted into the model to account for additional residual variance would be investigated (in each case the extra residual variance term was fitted for the subset with the highest residual variance). Apart from the extra random term, the model was the same as previously described. The significance of the extra random term was assessed (*Table 6.2*; See Chapter 5 for description of significance assessment). In each case (CLA, PLA_a, PLA_w, PLA_f and PLA_m) the extra random term was not significant therefore it was decided to proceed with the analysis with no additional random terms in the model to account for uneven residual variance.

Table 6.2 Significance of the random effects in the univariate model for interval to commencement of luteal activity postpartum (CLA), percentage of samples with luteal activity within 60 days postpartum using all (PLA_a), weekly (PLA_w), fortnightly (PLA_f) and monthly milk samples (PLA_m), with D being approximately chi-squared distributed with 1 degree of freedom (NS = non significant P>0.05)

Trait	Random terms	D	Significance
CLA	D1-F	0.00	NS
	Additive genetic	8.70	P<0.005
PLA_a	D1-F	0.96	NS
	Additive genetic	3.50	P=0.06
PLA_w	D1-F	0.06	NS
	Additive genetic	1.72	NS
PLA_f	D1-F	0.80	NS
	Additive genetic	2.32	NS
PLA_m	D1-F	0.00	NS
	Additive genetic	0.04	NS

6.2.4.2 Bivariate analysis of hormone and metabolite data with physiological fertility parameters

ASREML software (Gilmour *et al.,* 2006) was used to carry out bivariate analyses to assess the genetic and phenotypic (co) variation present between the hormone and metabolite data (FFA, glucose, GH, insulin and total IGF-I) with the fertility parameters (CLA, PLA_a, PLA_w, PLA_f and PLA_m). As in previous analyses, FFA, GH, insulin, IGF-I and CLA were $\log_{10}$ transformed to give approximately normally distributed residuals whilst glucose and PLA were approximately normally distributed already. The bivariate model contained both fixed and random terms specific to each parameter. Following the in depth study of the data (See Chapter 5 for the hormones and metabolites and above for physiological fertility parameters) extra random terms were fitted to account for additional residual variation within the hormone or metabolite in each dataset whereas

no extra terms were fitted to account for this in the physiological fertility parameters (*Table 6.2*). The bivariate model fitted to the data was:

Y_{ijnop} & $Z_{klmp} = \alpha + {}_{b1}P + {}_{b2}D + B_j + S_i + S.B + Y_k + S_l + F_m + R1_n + R2_o + A_p + \varepsilon_{ijklmnop}$

Where:

Y_{ijnop} = the hormone or metabolite concentration

Z_{klmp} = the physiological fertility parameter

the fixed effects in common to both Y and Z are:

α = intercept

${}_{b1}P$ = regression variable of sire percentage Holstein with coefficient *b1*

the fixed effects on Y only are:

${}_{b2}D$ = regression variable of age of animal in days with coefficient *b2*

B_i = the fixed effect of experimental batch (i = 1 to 74)

S_j = sex (j = female or male)

S.B = the sex.batch interaction

the fixed effects on Z only are:

Y_k = the effect of year of calving (k = 1 to 8)

S_l = the effect of season of calving (l = 1 to 4)

F_m = the effect of farm (m = 1 to 8)

the random effects applicable to Y only are:

$R1_n$ = the extra random term for extra residual variance (See Chapter 5 for details)

$R2_o$ = the extra random term for extra residual variance (See Chapter 5 for details)

and the random effects common to both Y and Z are:

A_p = breeding value ($N(0, \sigma_A^2\mathbf{A})$ where A is the numerator relationship matrix of animals available in the data)

$\varepsilon_{ijklmnop}$ = error term ($N(0, \sigma_E^2)$)

The bivariate analysis gave estimates for the genetic and phenotypic correlations between the hormone or metabolite data in each dataset (D1-F, D1-M and D2-F) with the physiological fertility parameter in (D1-F + D2-F combined) females. Since the

additional random variance terms, to account for differences in the residual variance in each subset, only affected the size of the phenotypic variance, for each hormone and physiological fertility parameter there was three phenotypic correlation estimates (one for each subset of data) and just one genetic correlation estimate. In each case, the difference between the three phenotypic correlation estimates was less than 0.02 and thus the range is displayed (lowest to highest; *Table 6.6 – 6.10*). In each bivariate analysis, heritability estimates were calculated: one for the physiological fertility parameter (D1-F + D2-F combined) and three for each metabolite or hormone (D1-F, D1-M and D2-F).

6.2.4.3 Regression of hormone and metabolites on sire PTAs

The univariate model previously described (Chapter 5) was used here with extra random terms fitted to account for additional residual variation within the hormone or metabolite in each dataset Each sire PTA (Section 6.2.3) was fitted into the univariate model separately, for the analyses of the hormone or metabolite data, as a regression variable as shown:

$$Y_{ijklm} = \alpha + {}_{b1}P + {}_{b2}D + {}_{b3}T + B_i + S_j + S.B + R1_k + R2_l + A_m + \varepsilon_{ijklm}$$

Where:

Y_{ijklm} = the hormone or metabolite concentration

the fixed effects are:

α = intercept

${}_{b1}P$ = regression variable of sire percentage Holstein with coefficient *b1*

${}_{b2}D$ = regression variable of age of animal in days with coefficient *b2*

${}_{b3}T$ = regression variable of sire PTA with coefficient *b3*

B_i = the fixed effect of experimental batch (i = 1 to 131)

S_j = sex (j = female or male)

S.B = the sex.batch interaction

and random effects are:

$R1_k$ = the extra random term for extra residual variance (in subset D1-M, D1-F & D2-F)

$R2_l$ = the extra random term for extra residual variance (in subset D1-M, D1-F & D2-F)

A_m = breeding value ($N(0, \sigma_A^2\mathbf{A})$ where A is the numerator relationship matrix of animals available in the data)

ε_{ijklm} = error term ($N(0, \sigma_E^2)$)

The regression coefficient for the sire PTA gave an indication of the genetic relationship between the sire PTA and the hormone or metabolite. The significance of the regression coefficient was calculated from the F statistic (calculated by ASREML). Approximate inferred genetic correlations were calculated by the following equation:

$$r_A = b * \frac{\sigma_a SirePTA}{\sigma_a Hormone / Metabolite}$$

Where:

r_A = the inferred genetic correlation

b = the regression coefficient for the sire PTA

σ_a = the genetic standard deviation of the sire PTA or the hormone or metabolite

The genetic standard deviations for the sire PTA, hormone and metabolite were obtained from several sources detailed in *Table 6.11*.

6.2.4.4 The significance of fixed and random effects

The standard error of the heritability, phenotypic and genetic correlation estimates were calculated using ASREML software (Gilmour *et al.*, 2006). The significance of the fixed and random effects in the univariate analysis of each physiological fertility parameter were assessed using Wald F statistics and the likelihood ratio test respectively. The log likelihood values are obtained from ASREML and the test statistic D approximates to the chi-squared distribution with 1 degree of freedom. It is calculated by:

$D = -2 [\log(R2) - \log(R1)]$

Where:

R1 = the log likelihood for the full model

R2 = the log likelihood for the restricted model i.e. with the random term in question removed

The significance of the genetic correlations were assessed by comparing the log likelihood of the full model to the log likelihood when the genetic covariance is fixed at zero. The D statistic is then calculated and approximates to the chi-squared distribution with one degree of freedom (as above).

The Wald F statistics produced by ASREML should be interpreted with caution. ASREML advises users that "the aim of the conditional Wald statistic is to facilitate inference for fixed effects and it is not meant to be prescriptive nor is it foolproof for every setting" (Gilmour *et al.*, 2006). Furthermore it is not possible to calculate the denominator degrees of freedom and so these are assumed to be large (approximately infinity) when analysing large datasets. For these reasons it is advised to use the conditional Wald F significance test to give an indication of the significance of fixed effects but particularly if terms are almost significant to leave them in the model.

6.3 RESULTS

6.3.1 Hormone and metabolite data

The number of animals, mean plasma concentration, standard deviation and standard error of glucose, FFA, GH, insulin and IGF-I for each dataset are shown in Chapter 5 (Section 5.3.1 *Table 5.5*).

6.3.2 Physiological fertility parameters

The number of records, mean, standard deviation and standard error for the fertility parameters calculated (CLA, PLA_a, PLA_w, PLA_f and PLA_m) are shown in *Table 6.3*. From *Table 6.3* it is evident that the females sampled in D2-F had shorter average CLA

and higher percentage of samples with luteal activity (PLA_a, PLA_w, PLA_f and PLA_m) which both may be indicative of earlier resumption of ovarian cyclicity. These differences could be due to genetic differences between animals and/or management differences between the two datasets and indeed the four dairy farms in D1-F and the four dairy farms in D2-F. However in the further analyses the effect of dairy farm was fitted to account for these differences.

Table 6.3 The number of animals, mean (before fitting the model), standard deviation (S.D.) and standard error (S.E.) of interval to commencement of luteal activity postpartum (CLA, days), percentage of samples with luteal activity within 60 days postpartum using all (PLA_a), weekly (PLA_w), fortnightly (PLA_f) and monthly milk samples (PLA_m; percentage) for dataset D1-F and D2-F.

Trait	Dataset	Number	Mean	S.D.	S.E.
CLA	D1-F	207	38.23	23.09	1.60
	D2-F	233	27.12	15.67	1.03
PLA_a	D1-F	220	39.12	23.61	1.59
	D2-F	123	51.70	22.78	2.05
PLA_w	D1-F	220	35.71	23.45	1.58
	D2-F	123	48.15	24.04	2.17
PLA_f	D1-F	220	32.74	25.35	1.71
	D2-F	123	42.44	23.24	2.10
PLA_m	D1-F	178	34.83	33.11	2.48
	D2-F	118	45.76	36.15	3.33

Figures 6.1 – 6.5 show the distribution of CLA, PLA_a, PLA_w, PLA_f and PLA_m in the females sampled. From these figures it can be seen that CLA has a skewed distribution (coefficient of skewness 2.11) and therefore was $\log_{10}$ transformed prior to further analysis. Although PLA_a, PLA_w, PLA_f and PLA_m (coefficients of skewness -0.155; 0.041; 0.290; 0.318 respectively) are not exactly normally distributed they were not transformed (See section 6.2.4.1). Similarly other studies have also not transformed

PLA data (Petersson *et al.,* 2006a; 2006b). Also evident from *Figures 6.1 – 6.5* is that subset D2-F has more animals with short CLA and high PLA than subset D1-F.

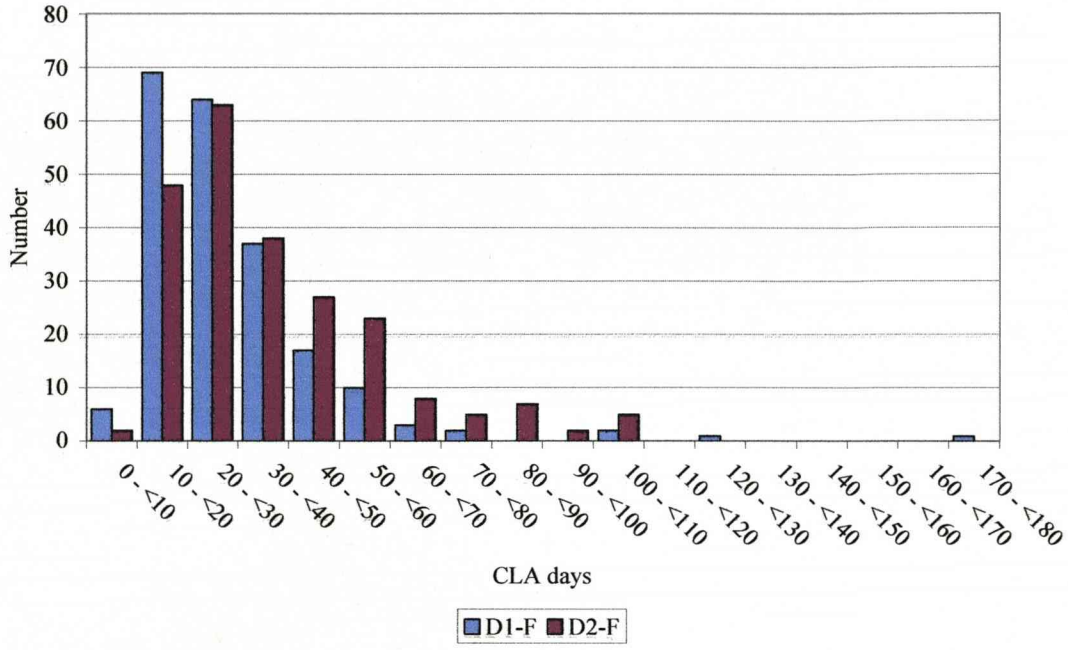

Figure 6.1 Distribution of the interval to commencement of luteal activity postpartum (CLA; days) in dataset D1-F and D2-F.

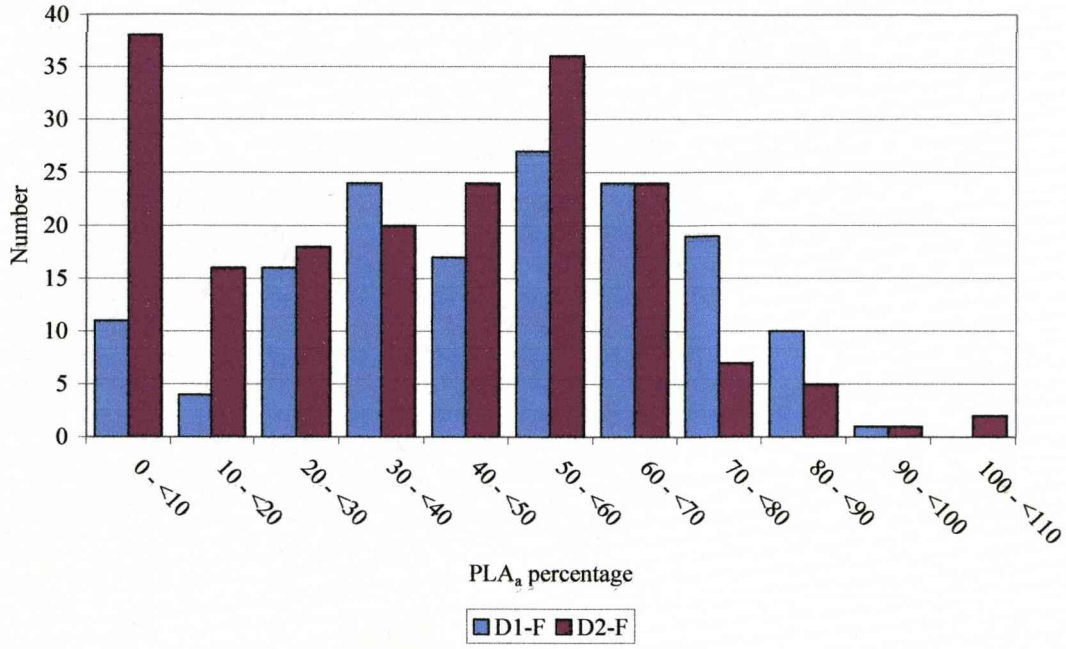

Figure 6.2 Distribution of the percentage of samples with luteal activity within 60 days postpartum using all milk samples (PLA_a; percentage) in dataset D1-F and D2-F.

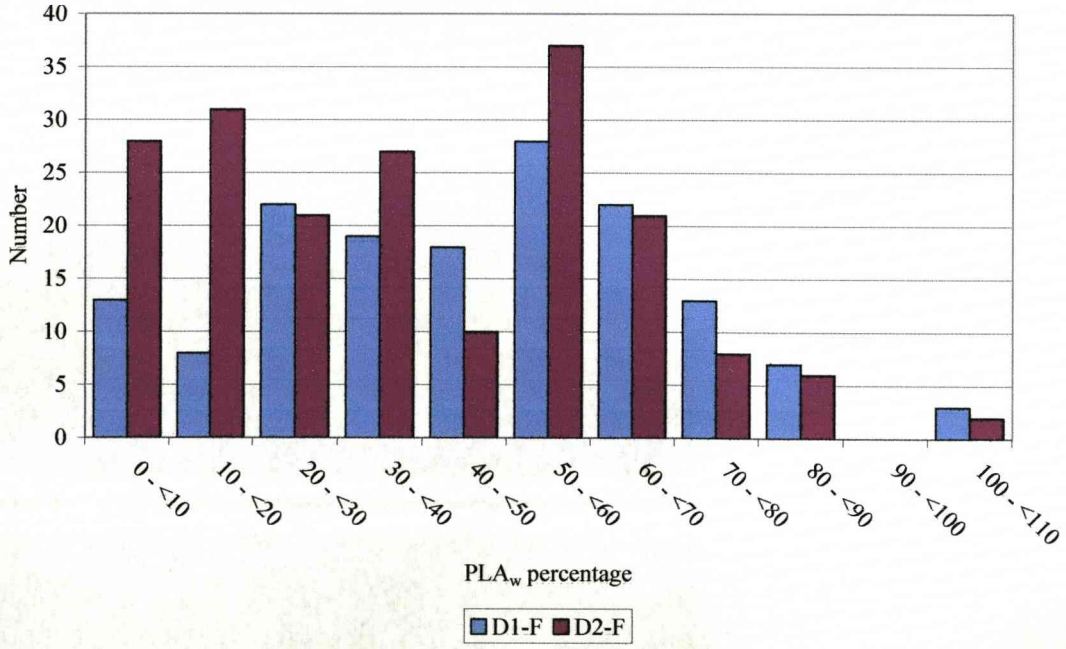

Figure 6.3 Distribution of the percentage of samples with luteal activity within 60 days postpartum using weekly milk samples (PLA_w; percentage) in dataset D1-F and D2-F.

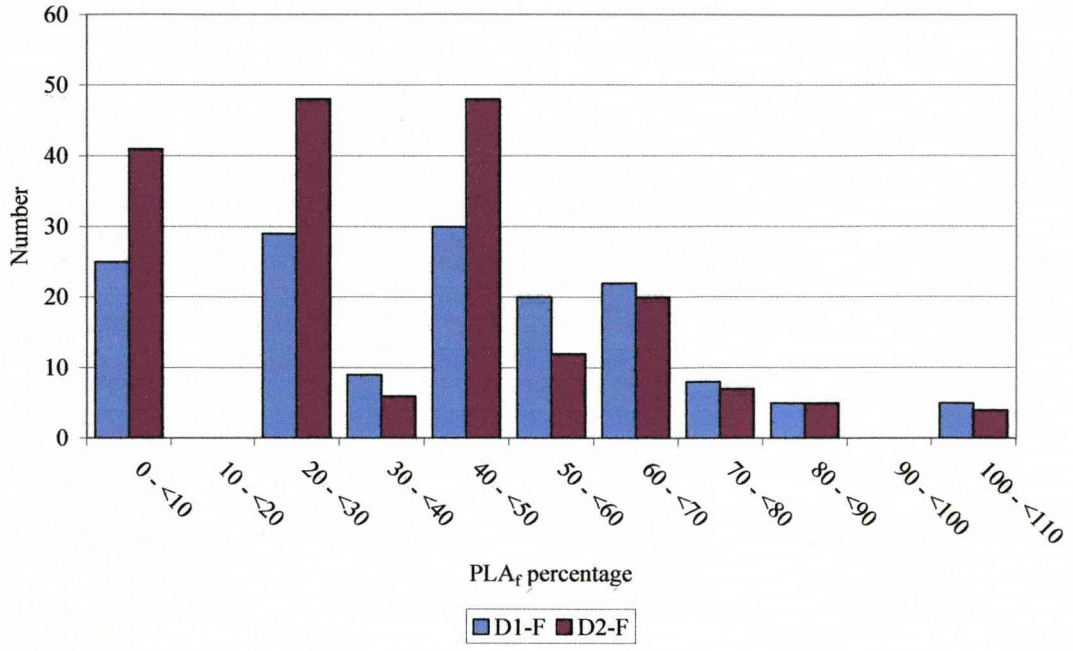

Figure 6.4 Distribution of the percentage of samples with luteal activity within 60 days postpartum using fortnightly milk samples (PLA_f; percentage) in dataset D1-F and D2-F.

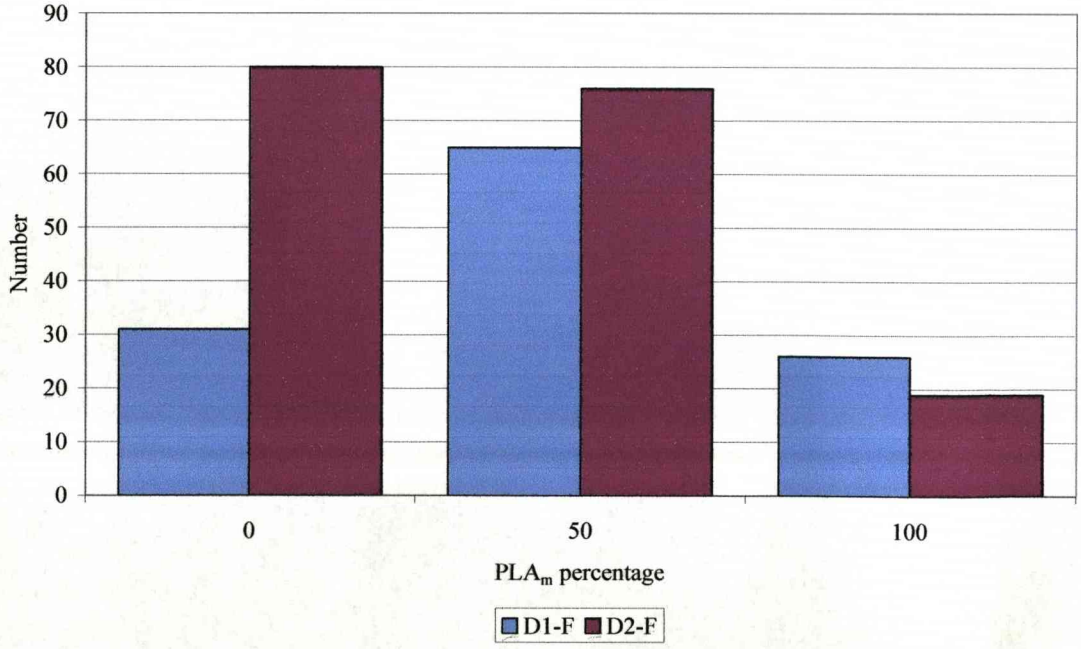

Figure 6.5 Distribution of the percentage of samples with luteal activity within 60 days postpartum using monthly milk samples (PLA_m; percentage) in dataset D1-F and D2-F.

6.3.3 Heritability of physiological fertility parameters

The estimated heritabilities plus standard errors and significance levels are shown in *Table 6.4*. The heritability of CLA is significant (P<0.005) whilst PLA_a is approaching significance (P = 0.08), PLA_w, PLA_f and PLA_m are not significant. The heritability of CLA was moderate and the heritabilities of PLA parameters decreased as the sampling frequency decreased.

Table 6.4 Estimated heritability (h^2) for interval to commencement of luteal activity postpartum (CLA), percentage of samples with luteal activity within 60 days postpartum using all (PLA_a), weekly (PLA_w), fortnightly (PLA_f) and monthly milk samples (PLA_m; percentage) plus standard errors (s.e.) for dataset D1-F + D2-F combined (NS = non significant P>0.05).

Trait	n	h^2	s.e. (h^2)	Significance
CLA	440	0.33	0.14	P<0.005
PLA_a	343	0.20	0.14	P=0.08
PLA_w	343	0.15	0.14	NS
PLA_f	343	0.08	0.12	NS
PLA_m	343	0.03	0.14	NS

6.3.4 Fixed and random effects

In the univariate analysis of the physiological fertility parameters the additive genetic, the only random term fitted, was significant in the case of CLA only (See *Table 6.4*).

The significance of the fixed effects for each fertility parameter are shown in *Table 6.5*. The fixed effect of sire percentage Holstein and farm were not significant in any of the fertility parameters. The calving year and season was significant in each analysis except for calving year in PLA_m and calving season in PLA_f analysis.

Table 6.5 The F test for significance of fixed terms fitted in each analysis. The denominator degrees of freedom (df) is assumed to be infinity due to the dataset being large (NS = non significant P>0.05)

Trait	Fixed term	Numerator df	F	Significance
CLA	Sire % Holstein	1	0.20	NS
	Calving year	7	3.00	P<0.005
	Calving season	3	9.41	P<0.001
	Farm	6	1.98	NS
PLA_a	Sire % Holstein	1	0.00	NS
	Calving year	5	3.60	P<0.005
	Calving season	3	8.73	P<0.001
	Farm	5	1.74	NS
PLA_w	Sire % Holstein	1	0.00	NS
	Calving year	5	3.60	P<0.005
	Calving season	3	8.73	P<0.001
	Farm	5	1.74	NS
PLA_f	Sire % Holstein	1	0.03	NS
	Calving year	5	2.69	P<0.025
	Calving season	3	1.58	NS
	Farm	5	0.96	NS
PLA_m	Sire % Holstein	1	0.01	NS
	Calving year	5	1.83	NS
	Calving season	3	4.34	P<0.005
	Farm	5	1.56	NS

6.3.5 Genetic and phenotypic correlations between hormone and metabolite data with physiological fertility parameters

Tables 6.6 - 6.10 show the genetic and phenotypic correlations (± standard error) of each physiological fertility parameter (CLA, PLA_a, PLA_w, PLA_f and PLA_m) with each hormone or metabolite. In each case the phenotypic correlations are close to zero, with a

small standard error, whereas the genetic correlations are greater in size but also with higher standard errors. In the PLA_m analysis the genetic correlation estimates are high and the accompanying standard errors are close to one or greater. This analysis should not be interpreted because a correlation can not exceed one, perhaps with a larger dataset this fertility parameter could be analysed.

Table 6.6 Genetic (r_A) and phenotypic (r_P) correlations (± standard error) of the hormones and metabolites with the interval to commencement of luteal activity postpartum (CLA log_{10} units; unless otherwise stated correlations are not significant P>0.05)

Trait	N	r_A (± s.e.)	r_P (± s.e.)
Glucose	432	-0.37 ± 0.34	-0.03 ± 0.04 to -0.05 ± 0.06
FFA	432	0.03 ± 0.35	0.01 ± 0.04 to 0.01 ± 0.06
GH	435	0.03 ± 0.35	0.01 ± 0.05 to 0.01 ± 0.06
Insulin	435	-0.36 ± 0.35	-0.05 ± 0.05 to -0.08 ± 0.08
IGF-1	427	-0.07 ± 0.26	-0.05 ± 0.04 to -0.08 ± 0.07

Table 6.7 Genetic (r_A) and phenotypic (r_P) correlations (± standard error) of the hormones and metabolites with the percentage of samples with luteal activity within 60 days postpartum using all samples (PLA_a; unless otherwise stated correlations are not significant P>0.05)

Trait	N	r_A (± s.e.)	r_P (± s.e.)
Glucose	335	-0.18 ± 0.45	-0.00 ± 0.05 to -0.00 ± 0.07
FFA	335	-0.34 ± 0.45	0.00 ± 0.05 to 0.01 ± 0.08
GH	338	0.13 ± 0.44	-0.01 ± 0.05 to -0.01 ± 0.06
Insulin	338	0.13 ± 0.35	0.03 ± 0.05 to 0.04 ± 0.08
IGF-1	331	-0.14 ± 0.35	0.05 ± 0.05 to 0.08 ± 0.09

Table 6.8 Genetic (r_A) and phenotypic (r_P) correlations (± standard error) of the hormones and metabolites with the percentage of samples with luteal activity within 60 days postpartum weekly samples (PLA_w; unless otherwise stated correlations are not significant P>0.05)

Trait	N	r_A (± s.e.)	r_P (± s.e.)
Glucose	335	-0.23 ± 0.50	0.01 ± 0.05 to 0.02 ± 0.06
FFA	335	-0.57 ± 0.47	-0.01 ± 0.05 to -0.01 ± 0.07
GH	338	0.08 ± 0.49	-0.04 ± 0.05 to -0.05 ± 0.06
Insulin	338	0.21 ± 0.54	0.02 ± 0.05 to 0.03 ± 0.08
IGF-1	331	-0.12 ± 0.41	0.02 ± 0.05 to 0.03 ± 0.08

Table 6.9 Genetic (r_A) and phenotypic (r_P) correlations (± standard error) of the hormones and metabolites with the percentage of samples with luteal activity within 60 days postpartum using fortnightly samples (PLA_f; unless otherwise stated correlations are not significant P>0.05)

Trait	N	r_A (± s.e.)	r_P (± s.e.)
Glucose	335	0.17 ± 0.70	0.02 ± 0.05 to 0.03 ± 0.07
FFA	335	-0.82 ± 0.61	-0.01 ± 0.05 to -0.02 ± 0.07
GH	338	0.00 ± 0.63	-0.02 ± 0.05 to -0.02 ± 0.06
Insulin	338	0.76 ± 0.77	0.03 ± 0.05 to 0.04 ± 0.08
IGF-1	331	0.14 ± 0.56	0.06 ± 0.05 to 0.10 ± 0.09

Table 6.10 Genetic (r_A) and phenotypic (r_P) correlations (± standard error) of the hormones and metabolites with the percentage of samples with luteal activity within 60 days postpartum using monthly samples (PLA_m; unless otherwise stated correlations are not significant P>0.05)

Trait	N	r_A (± s.e.)	r_P (± s.e.)
Glucose	288	0.01 ± 0.95	-0.06 ± 0.05 to -0.08 ± 0.07
FFA	288	0.01 ± 1.25	-0.05 ± 0.05 to -0.09 ± 0.08
GH	291	-0.51 ± 1.93	0.02 ± 0.05 to 0.03 ± 0.06
Insulin	291	0.20 ± 1.46	0.04 ± 0.06 to 0.06 ± 0.10
IGF-1	284	-0.80 ± 0.66	-0.11 ± 0.05 to -0.20 ± 0.09

6.3.6 Regressions of the hormone and metabolite on the sire PTAs

Table 6.11 shows the heritability estimates and the genetic standard deviations for the glucose, FFA, GH, insulin, IGF-1 and the sire PTAs. *Table 6.12 - 6.16* show the genetic regression coefficients of glucose, FFA, GH, insulin and IGF-I data on the sire PTAs and the approximate inferred genetic correlations.

Table 6.11 Heritability estimates, genetic standard deviation (σ_a) and reference for each estimate.

Trait	h^2	σ_a	Reference
Glucose	0.13 – 0.23	0.2514	Chapter 5
FFA	0.09 – 0.25	0.1257	
GH	0.13 – 0.18	0.2606	
Insulin	0.10 – 0.22	0.1879	
IGF-1	0.21 – 0.66	0.2814	
305d milk yield kg	0.59	810.70	R. Mrode, personal communication
305d fat yield kg	0.48	25.20	
305d protein yield kg	0.55	21.86	
Fat percentage	0.68	0.32	
Protein percentage	0.68	0.14	
PIN	Not calculated	Not calculated	
PLI	Not calculated	Not calculated	
Fertility index	Not calculated	Not calculated	
Non return rate 56d	0.018	0.06	Wall *et al.*, 2003b
Calving interval	0.033	8.95	
DIM first AI	0.035	5.03	
N° services per conception	0.020	0.14	
Condition score	0.237	0.68	

The regression coefficients and approximate genetic correlations of the sire PTAs with glucose and IGF-1 were all non-significant (*Table 6.12 & 6.16*). The regression coefficients of protein percentage with FFA and insulin concentration were significant (*Table 6.13 & 6.15*). Similarly the regression coefficient of GH on the fertility index PTA was significant and negative whereas, the approximate genetic correlation between GH and sire PTA for calving interval was positive and significant (*Table 6.14*). Other regression coefficients and approximate genetic correlations were non significant (P=>0.10; *Table 6.12 – 6.16*).

Table 6.12 Genetic regression coefficients (b ± standard error) of glucose on the sire PTAs, plus the significance of b and the approximate genetic correlation (r_A)

PTA	***b* ± standard error**	**Significance**	**Approximate r_A**
305d milk yield kg	0.0001508 ± 0.0001116	NS	0.49
305d fat yield kg	-0.0000388 ± 0.003661	NS	0.00
305d protein yield kg	0.002998 ± 0.003805	NS	0.26
Fat percentage	-0.3225 ± 0.2341	NS	-0.42
Protein percentage	-0.7227 ± 0.5600	NS	-0.39
PIN	0.0002365 ± 0.001391	NS	----
PLI	0.0001800 ± 0.001424	NS	----
Fertility index	0.003824 ± 0.007824	NS	----
Non return rate 56d	0.002569 ± 0.01379	NS	0.00
Calving interval	-0.009051 ± 0.008158	NS	-0.32
DIM first AI	-0.001069 ± 0.02022	NS	-0.02
N° services per conception	0.4116 ± 0.8638	NS	0.23
Condition score	0.02552 ± 0.03348	NS	0.07

Table 6.13 Genetic regression coefficients (b ± standard error) of FFA on the sire PTAs, plus the significance of b and the approximate genetic correlation (r_A)

PTA	b ± standard error	Significance	Approximate r_A
305d milk yield kg	0.00001006 ± 0.00005677	NS	0.06
305d fat yield kg	-0.00008436 ± 0.001853	NS	-0.02
305d protein yield kg	-0.002340 ± 0.001885	NS	-0.41
Fat percentage	-0.03580 ± 0.1205	NS	-0.09
Protein percentage	-0.7982 ± 0.2789	P<0.005	-0.87
PIN	-0.0009706 ± 0.0006919	NS	----
PLI	-0.0005998 ± 0.0007044	NS	----
Fertility index	0.003459 ± 0.004068	NS	----
Non return rate 56d	0.005364 ± 0.007292	NS	0.00
Calving interval	-0.002613 ± 0.004241	NS	-0.19
DIM first AI	-0.005894 ± 0.01265	NS	-0.24
N° services per conception	-0.1614 ± 0.5589	NS	-0.18
Condition score	0.009926 ± 0.01673	NS	0.05

Table 6.14 Genetic regression coefficients (b ± standard error) of GH on the sire PTAs, plus the significance of b and the approximate genetic correlation (r_A)

PTA	b ± standard error	Significance	Approximate r_A
305d milk yield kg	-0.00005565 ± 0.0001232	NS	-0.17
305d fat yield kg	0.0002864 ± 0.004066	NS	0.03
305d protein yield kg	-0.001399 ± 0.004251	NS	-0.12
Fat percentage	0.1089 ± 0.2568	NS	0.14
Protein percentage	0.1092 ± 0.6216	NS	0.06
PIN	-0.00009583 ± 0.001561	NS	----
PLI	-0.001943 ± 0.001489	NS	----
Fertility index	-0.01448 ± 0.008498	P<0.10	----
Non return rate 56d	-0.01994 ± 0.1480	NS	0.00
Calving interval	0.01864 ± 0.008682	P<0.05	0.64
DIM first AI	0.004676 ± 0.02140	NS	0.09
N° services per conception	0.6795 ± 0.9519	NS	0.37
Condition score	0.01115 ± 0.03478	NS	0.03

Table 6.15 Genetic regression coefficients (b ± standard error) of insulin on the sire PTAs, plus the significance of b and the approximate genetic correlation (r_A)

PTA	b ± standard error	Significance	Approximate r_A
305d milk yield kg	-0.00002218 ± 0.00009491	NS	-0.10
305d fat yield kg	-0.001515 ± 0.003193	NS	-0.20
305d protein yield kg	0.003285 ± 0.003345	NS	0.38
Fat percentage	0.1177 ± 0.1992	NS	0.20
Protein percentage	1.127 ± 0.4626	P<0.025	0.82
PIN	0.001669 ± 0.001233	NS	----
PLI	0.001427 ± 0.001254	NS	----
Fertility index	-0.003190 ± 0.006739	NS	----
Non return rate 56d	-0.001509 ± 0.01202	NS	0.00
Calving interval	0.01057 ± 0.007099	NS	0.50
DIM first AI	0.01230 ± 0.01454	NS	0.33
N° services per conception	0.3485 ± 0.6349	NS	0.26
Condition score	0.0006452 ± 0.02862	NS	0.00

Table 6.16 Genetic regression coefficients (b ± standard error) of IGF-1 on the sire PTAs, plus the significance of b and the approximate genetic correlation (r_A)

PTA	b ± standard error	Significance	Approximate r_A
305d milk yield kg	-0.000002834 ± 0.00008755	NS	-0.01
305d fat yield kg	-0.002072 ± 0.002843	NS	-0.19
305d protein yield kg	0.00007138 ± 0.002983	NS	0.01
Fat percentage	-0.1051 ± 0.1829	NS	-0.12
Protein percentage	0.05033 ± 0.4285	NS	0.02
PIN	-0.0003152 ± 0.001082	NS	----
PLI	-0.0001537 ± 0.001097	NS	----
Fertility index	0.006778 ± 0.005973	NS	----
Non return rate 56d	0.01748 ± 0.01073	NS	0.00
Calving interval	0.008519 ± 0.006550	NS	0.27
DIM first AI	0.02565 ± 0.01643	NS	0.46
N° services per conception	-0.5322 ± 0.6874	NS	-0.26
Condition score	-0.03539 ± 0.02589	NS	0.09

6.4 DISCUSSION

This study has found that significant additive genetic variation is responsible for a proportion of the phenotypic variation in CLA ($P<0.05$) and PLA_a ($P=0.08$) in first lactation Holstein-Friesian heifers. The sire PTAs for protein percentage, fertility index and DIM till first AI had significant regression coefficients and approximate genetic correlations with FFA, GH and insulin. Other regressions were non significant. Furthermore, genetic correlations between glucose, FFA, GH, insulin and IGF-1 concentrations with CLA, PLA_a, PLA_w, PLA_f and PLA_m although not significant were largely moderate, with only few exceptions, whilst phenotypic correlations were close to zero.

6.4.1 Size limitations of the analyses

Before the results of this chapter are discussed it is important to first highlight the main limitation of these analyses. The number of records used for the analyses of the hormone and metabolite concentrations is small (D1-M + D1-F + D2-F n = 1077) and even fewer records were available for the physiological fertility parameters (D1-F + D2-F n = 440 for CLA; n = 343 for PLA). Furthermore, the number of animals that had sire PTAs was also small particularly for the fertility PTAs (Milk production PTAs n = 1035; fertility PTAs n = 563 to 983). The small size of datasets often leads to non-significant genetic parameter estimates with large standard errors. The optimal design theory, described by Robertson (1959; Chapter 3), was used to determine the experimental design of the data collected (D1 and D2) however the cost and the time taken for such experiments in cattle often leads to a reduced number of animals and records than originally planned. The results to be discussed in this chapter are mainly non-significant with large standard errors therefore interpretation must be with caution. The direction of the genetic and phenotypic correlations will be interpreted as an indication of possible relationship rather than the size of the correlation due to the low accuracy and size of data. Similarly, the heritabilities must be interpreted carefully as only an indication of possible genetic variation present in the trait which may be confirmed, larger or smaller in a larger dataset (e.g. Darwash *et al.*, 1997a; Veerkamp *et al.*, 2000; Royal *et al.*, 2002; Petersson *et al.*, 2007).

6.4.2 Heritability of physiological fertility parameters

This study has shown that moderate and significant additive genetic variance is present in CLA (h^2 ± standard error 0.33 ± 0.14, $P<0.05$) in Holstein-Friesian heifers during their first lactation. The heritability of PLA_a was moderate and approaching significance (h^2 ± standard error 0.20 ± 0.14, $P=0.08$) whilst the heritabilities of PLA_w, PLA_f and PLA_m were non significant and low (heritability range 0.03 to 0.15; *Table 6.4*).

The heritability of CLA is similar, but somewhat higher, to those reported in the literature. A study by Darwash *et al.* (1997a) analysed CLA records for 1737 lactations of 1137 British Friesian cows (1975 – 1982) and found this trait, when log transformed, to be moderately heritable at 0.21. More recently Royal *et al.* (2002) analysed milk progesterone records from 1212 lactations from 1080 Holstein-Friesian cows (1996 - 1999) and found CLA to be moderately heritable (h^2 ± standard error, 0.16 ± 0.05). A similar heritability estimate for CLA was found by Veerkamp *et al.* (2000) in first lactation Holstein-Friesian heifers in the Netherlands (n = 329, h^2 ± standard error, 0.16 ± 0.10). A pooled and weighted estimate for the heritability of CLA was reported by Royal in 1999 as similar (h^2 ± standard error, 0.20 ± 0.05) to those above. The estimate in the present study is higher than previously reported however the dataset is small (n = 440).

This study found the PLA fertility measures, first characterised by Petersson *et al.* (2006a; 2006b), to have lower heritabilities than previously reported. Petersson *et al.* (2007) analyzed milk progesterone records, previously analyzed by Royal *et al.* (2002), from 1212 lactations (n = 1080 British Holstein-Friesian) and found each PLA measure to be heritable (h^2 ± standard error, PLA_a 0.30 ± 0.06; PLA_w 0.25 ± 0.06; PLA_f 0.20 ± 0.06; PLA_m 0.14 ± 0.06) and estimates decreased as the sampling frequency was reduced (Petersson *et al.*, 2007).

The differences between the heritability estimates in the present study for both CLA and PLA measures could be due to the small number of records (See section 6.4.1; n = 440 CLA; n = 343 PLA). Additionally, it is possible that in this study there is a greater degree of resemblance between related animals in the two datasets D1-F and D2-F thus increasing the additive genetic variance. Conversely in this study there could be a greater degree of resemblance between all animals particularly within D1-F and D2-F which would reduce the total phenotypic variance and thus increase the heritability estimate. Higher or lower gene frequencies, compared to other cattle populations (e.g. Darwash *et al.*, 1997a; Veerkamp *et al.*, 2000; Royal *et al.*, 2002; Petersson *et al.*, 2007), can also affect the additive genetic variance and thus the heritability estimates. In

dataset D1-F, animals have been bred centrally at a breeding company therefore the breeding program is the same for animals in D1-F and furthermore animals are likely to show a greater degree of relatedness, both of which may increase the proportion of phenotypic variance that can be attributed to additive genetic variation.

6.4.3 Fixed and random effects

The random effect of additive genetic variance fitted for each physiological fertility parameter was only significant in the case of CLA. The non significance for the PLA fertility parameters is likely due to the reduced size of the dataset in comparison to CLA (See section 6.4.1).

The fixed effects of sire percentage Holstein was not significant in any analyses. As discussed in Chapter 5 the sire percentage Holstein was fitted to account for breed differences, i.e. percentage Holstein genes, between the animals over the period of testing. The difference in sire percentage Holstein was small and obviously had no significant effect on the physiological fertility measures in this study. However, it is recognised that the percentage of North American Holstein genes in the UK herd has increased greatly over the last 40 years as discussed in Chapter 3. This increase in Holstein genes, in addition to intense selection, is responsible for the increase in milk production but unfortunately it has also, in part, caused the low fertility seen at present (average conception rate to first service 37.1 % n = 2471, Mayne *et al.*, 2002; 39.7 % n = 714, Royal *et al.*, 2000a). Hoekstra *et al.* (1994) reported a significant effect due Holstein genes, in a Dutch Holstein-Friesian crossbred herd (n = 13480), on both production and fertility. Similarly, Evans *et al.* (2006b) reported that calving interval increased as percentage Holstein genes increased.

The fixed effect of farm (n = 8) was not significant in any analyses. This implies that management effects on fertility, specific to individual farms, in these analyses had no significant effect on the physiological fertility measures in the present study. Work by Royal *et al.* (2002) found that farm had a significant effect on pregnancy rate to first

service and interval to first service yet not on CLA, length of luteal phase or on persistent corpus luteum suggesting that traditional fertility parameters rather than those based on milk progesterone measurement are more sensitive to farm specific effects. A study by Windig *et al.* (2005) examined the effect of herd environment on health and fertility in 3904 herds in The Netherlands. The results showed that herd environment affected health and fertility though the effects were due to four components of herd environment: average production per cow, average fertility, farm size and relative performance i.e. good (poor) health and fertility despite high (low) production. It is likely that farm does affect fertility through differences in management, herd size, general health and fertility and if differences in these components are small between farms then farm will not appear to affect fertility as perhaps has been the case in the present study.

The year of calving was significant for each physiological fertility parameter (CLA, PLA_a, PLA_w and PLA_f) except PLA_m. The year of calving accounts for many things specific to certain years e.g. diet, management, staff, weather and it would appear that some of these may have contributed to the significance of year of calving. Additionally, the significant effect of calving year could be due to the steady decline of fertility levels in the dairy cow around the world (e.g. UK, Royal *et al.,* 2000a; Belgium, Opsomer *et al.*, 1998; USA, Lucy, 2001; Ireland, Evans *et al.*, 2006; Spain, López-Gatius, 2003; the Netherlands, Ouweltjes *et al.*, 1996). However the animals studied calved over a relatively short period of 8 years between 1998 and 2006 and the present dataset is small (n = 440). The fixed effect of calving season was significant in the analyses of CLA, PLA_a, PLA_w and PLA_m with animals having a shorter CLA and higher PLA in seasons 3 (June, July and August) and 4 (September, October and November) yet the opposite in season 2 (March, April and May). Differences due to season of calving were also reported by Royal *et al.* (2002) who found cows calving in season 2 had the longest CLA whereas in season 3 and 4 average CLA was shortest. The same pattern of average CLA in different calving seasons was reported by Darwash *et al.* (1997b). These affects are possibly due to differences in diet due to season with spring grass being of poorer quality compared to summer and autumn grass and/or due to photoperiod. However the

effect of diet is only relevant to animals in D2-F as those in D1-F were housed all year with no turnout and therefore differences in diet due to season would be minimal.

6.4.4 Physiological fertility parameters

The average CLA in the present study is longer in D1-F (average days ± standard error, 38.23 ± 1.60) than D2-F (27.12 ± 1.03) indicating earlier resumption of luteal activity in D2-F. CLA was assessed in animals in D2-F calving between 1998 to 2001 whereas animals in D1-F calved between 2004 to 2006. Therefore differences in CLA could be partly due to year. The average CLA in D1-F is longer than that reported by Darwash *et al.* (1997a), Royal *et al.* (2002) and Veerkamp *et al.* 2000 whereas D2-F is comparable to the literature. Petersson *et al.* (2006a; 2007), using the same data described by Royal *et al.* (2002), reported average PLA_a that was midway between D1-F and D2-F in the present study. One point to consider in the interpretation of PLA measures is that whilst cows in D2-F were milk sampled until 80 days postpartum cows in D1-F were more often only milk sampled until CLA occurred and those that were sampled upto and beyond 60 days postpartum had problems resuming normal ovarian cyclicity.

Another consideration is that the four farms in D1-F were the commercial herds associated with a dairy breeding company. These herds were large, high yielding herds and perhaps at a phenotypic level there aim was more focused on yield rather than fertility, than other farms such as those in D2-F. The affect of herd environment on fertility was examined by Windig *et al.* (2005) who reported that high production herds had fewer average days till first service postpartum but with lower success compared to lower yielding herds whereas in large herds drops in production were less frequent and fertility was better than in smaller herds. The difference in CLA and PLA between D1-F and D2-F may not be due to the association of D1-F with a breeding company but more likely due a combination of factors including differences in average milk yield, herd size and management factors (that were minimized within D1-F due to the common management decisions etc).

6.4.5 Genetic and phenotypic correlations between hormone and metabolite data with physiological fertility parameters

As discussed in section 6.4.1 the direction of correlations will only be interpreted due to the size of the dataset, non-significance of estimates and large standard errors. Furthermore, these results should be interpreted with care as further estimation of these parameters in larger datasets is needed. *Figure 6.6 & 6.7* are schematic representations of the direction of genetic and phenotypic correlations respectively between CLA, PLA_a, PLA_w and PLA_f with the glucose, FFA, GH, insulin and IGF-1 (PLA_m is not interpreted, see section 6.3.5).

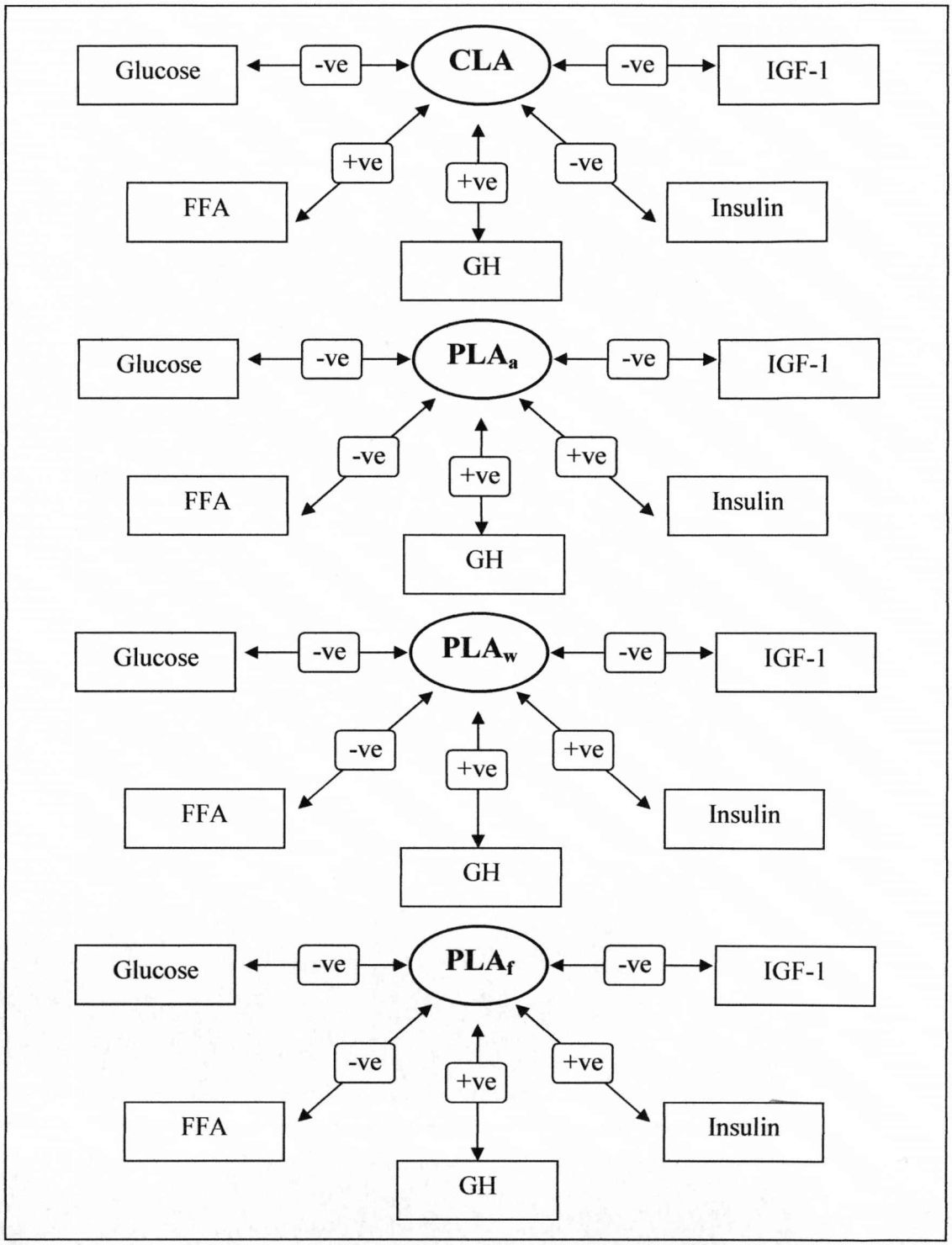

Figure 6.6 Illustration of the positive (+ve) and negative (-ve) genetic correlations between physiological fertility parameters (CLA, PLA_a, PLA_w and PLA_f) with glucose, FFA, GH, insulin and IGF-1.

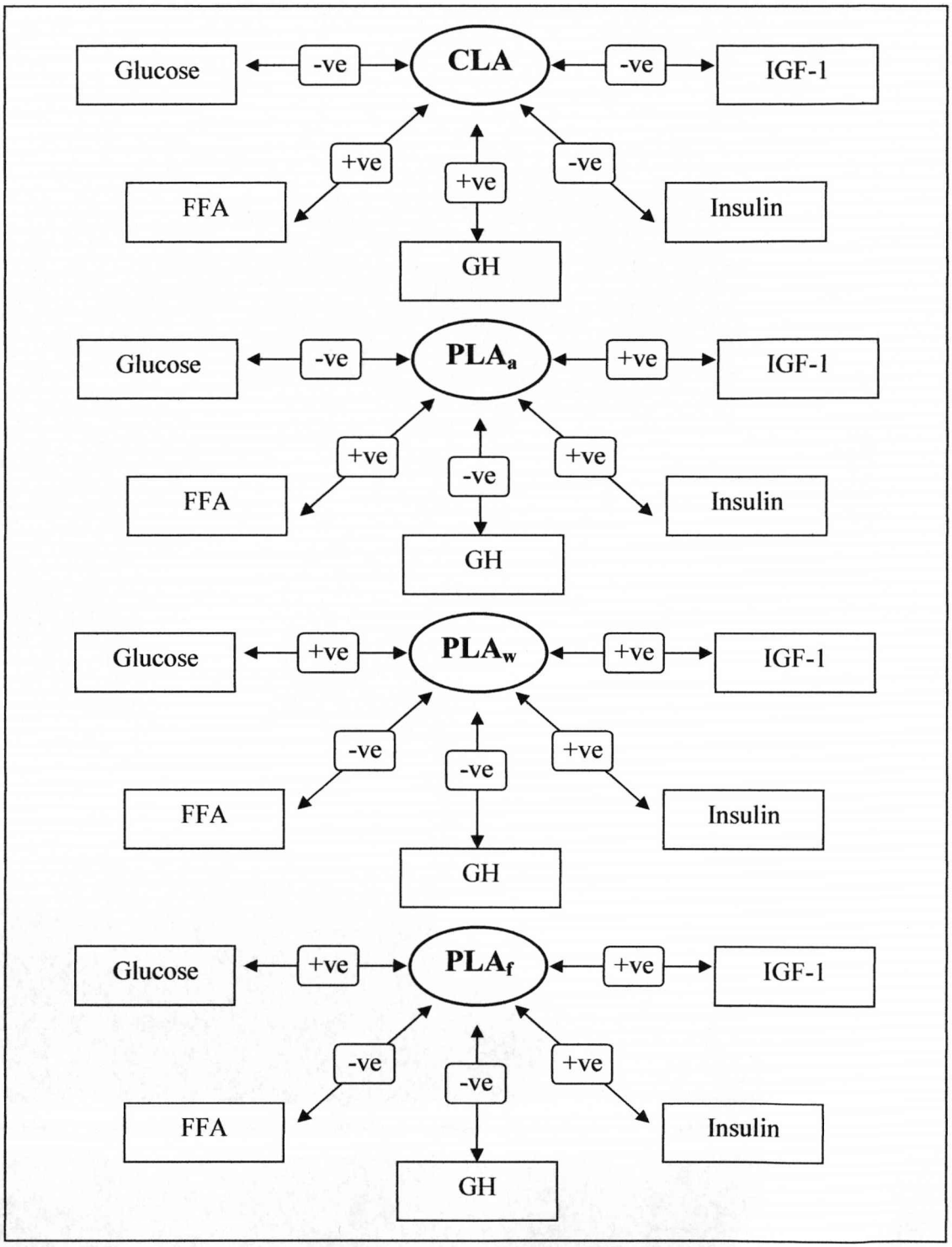

Figure 6.7 Illustration of the positive (+ve) and negative (-ve) phenotypic correlations between physiological fertility parameters (CLA, PLA_a, PLA_w and PLA_f) with glucose, FFA, GH, insulin and IGF-1.

6.4.5.1 Correlations between CLA with hormones and metabolites

The genetic correlations of glucose and insulin with CLA (*Table 6.6*) were large and negative and although the standard errors were large the addition or subtraction of 1 x the standard error would have no effect on the direction of the correlation. Furthermore, although these genetic correlations were not significant (P>0.05) this is perhaps due to the small size of data (Section 6.4.1; n = 440 approximately). The genetic correlation between CLA with FFA, GH and IGF-1 were all close to zero and non-significant (*Table 6.6*). In addition to this, the phenotypic correlations were all close to zero and non-significant (*Table 6.6*).

The genetic correlations between CLA with glucose and insulin suggest that a high concentration of glucose or insulin in female calves at 4 months of age is genetically related to a shorter CLA as first lactation heifers (*Figure 6.6*). Analyses in Chapter 5 found that concentrations of insulin and glucose are highly genetically correlated (Chapter 5 *Table 5.9*; genetic correlation ± standard error, 0.67 ± 0.30) in 4 month old calves and therefore it is reasonable that insulin and glucose would show a similar genetic correlation to CLA. Although, to the authors knowledge, there have been no genetic studies linking hormones and metabolites in calves with fertility, it is known that high concentrations of glucose and insulin particularly in the postpartum period are beneficial to follicular growth and fertility. The exact role of glucose in follicular growth is not clear but during periods of dietary restriction, such as postpartum negative energy balance (NEB), lowered plasma glucose concentration is thought to lead to reduced LH pulsatility through GnRH (Diskin *et al.,* 2003). Insulin is important in that it stimulates steroid production by the growing follicle, granulosa cell proliferation, and it stimulates progesterone production by the corpus luteum (Langhout *et al.*, 1991; Mihm *et al.*, 2002; Webb *et al.*, 2004).

Increased insulin concentrations postpartum were shown to be beneficial to reproductive function through work by Gong *et al.* (2002). This study examined the effects of feeding a diet designed to increase plasma insulin concentrations in Holstein-Friesian

heifers for 50 days postpartum. High (n = 10 control diet; n = 10 high insulin diet) and low (n = 10 control diet; n = 10 high insulin diet) genetic merit for milk yield lines were fed either a control diet which provided energy and protein for maintenance and milk production or a high insulin diet designed to increase acetate or propionate production in the rumen and lead to higher insulin response to feeding. The high insulin diet caused a small increase in milk yield compared to control diet of the same genetic merit though this was not significant. Plasma insulin concentration was higher in the low genetic merit line than the high genetic merit line and within each genetic merit group in the high insulin diet (*Figure 6.8*).

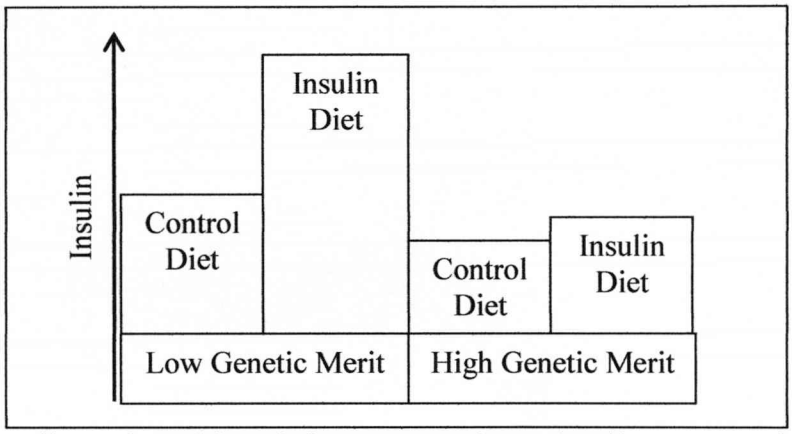

Figure 6.8 Illustration of the relative average plasma insulin concentration during the period of feeding a control diet and diet designed to increase insulin in low and high genetic merit for milk yield cows (Gong *et al.,* 2002)

Various fertility measures (interval from calving to first service, interval from calving to conception, conception rate to first service and overall conception rate) were improved by feeding a high insulin diet (in both genetic merit groups) and furthermore, fertility was better in the low genetic merit than high genetic merit groups irrespective of diet (Gong *et al.*, 2002). It is well recognised that altered hormone and metabolite concentrations as a result of NEB postpartum are the main cause of poor resumption of ovarian cyclicity and fertility (Butler, 2000; Roche *et al.*, 2000; Diskin *et al.*, 2003). It is possible that female calves who exhibit a higher concentration of glucose and insulin at 4 months of age are better able to withstand some of the effects of NEB postpartum and

indeed have higher concentrations of glucose and insulin and a shorter interval to luteal activity postpartum. Taylor *et al.* (2004) reported a phenotypic link between metabolic profiles in female calves (6 months) with ovarian function postpartum (assessed by milk progesterone sampling). That study found glucose concentrations at 6 months of age to be negatively associated with concentrations of glucose after calving (week -1, 1, 3, 5 and 8 postpartum; $P<0.05$) however the number of animals was low ($n = 32$). Furthermore, there was no significant association between glucose or insulin in the prepubertal calf with milk progesterone profile (normal, persistent corpus luteum or delayed ovulation; see Chapter 1 for description).

The genetic correlation estimated in the present study between glucose and CLA (negative i.e. high glucose related to short CLA; *Figure 6.6*) is in conflict with that found in Danish calves (negative i.e. high glucose related to low fertility index; Chapter 4). Although it is not advisable to directly compare CLA with the Danish fertility index because they are very different measures, they are both genetically correlated to traditional fertility measures such as calving interval. In Chapter 4, concentrations of glucose following an overnight fast in male 9 month old calves was negatively genetically correlated to female fertility index (female fertility estimated breeding values of the males as sires) suggesting that a low concentration of glucose led to female offspring with better fertility index. It is likely that the difference in this correlation between glucose and the fertility index in Danish and the CLA in UK calves is due to the effect of fasting. A high concentration of glucose during normal feed allowance and a low concentration of glucose after some hours (22 hours) of feed deprivation are genetically correlated to shorter CLA and higher Danish fertility index respectively as heifers. Glucose is relatively stable in cows and concentrations are kept within an upper and lower amount by the alternating secretion of insulin and glucagon (Jiang and Zhang, 2003). Animals with a lower critical glucose concentration, below which glucagon is released, perhaps are better able to maintain body reserves during NEB (See Chapter 4).

In contrast to the finding here, Chapter 4 also found that insulin concentration, in 9 month old males following a fast, was not genetically correlated to the Danish fertility index.

6.4.5.2 Correlations between PLA measures with hormones and metabolites

The phenotypic correlations between all PLA measures (PLA_a, PLA_w, PLA_f and PLA_m) with the hormones and metabolites were close to zero and non-significant (*Figure 6.7*). This is probably due to the small dataset (n = 440; Section 6.4.1).

The physiological fertility measure PLA_a was found to be negatively genetically correlated with glucose, FFA and IGF-1 and positively genetically correlated with GH and insulin (*Figure 6.6*). However, these correlations were not significant and their standard errors were larger than the estimates themselves. This is partly due to the small size of the dataset (n = 440) and perhaps these correlations should be used only to give an indication of the direction of relationship rather than the magnitude. The PLA fertility measures are complicated to interpret (Chapter 1.6.2); the ideal PLA has been suggested as being intermediate (Petersson *et al.*, 2007). Low PLA has been found to be genetically associated with delayed ovulation postpartum whilst high PLA associated with persistent corpus luteum (Petersson *et al.*, 2007).

The negative genetic correlations, in the present study (*Figure 6.6*), suggest that high glucose, FFA and IGF-1 in 4 month old calves lead to heifers with a low percentage of samples with luteal activity during the first 60 days postpartum. Low concentrations of glucose and IGF-1 are seen during NEB and are associated, in addition to other changes, with a poor resumption of luteal activity (Butler, 2000). Therefore the negative genetic correlation found here between glucose and IGF-1 with PLA_a is somewhat surprising and perhaps reflective of the difficulties in the interpretation of PLA levels (Chapter 1.6.2). The negative genetic correlation between FFA and glucose with PLA_a is supportive of the findings in Chapter 4 where genetic correlations indicated that male

calves with high glucose and FFA following overnight fast at 9 months of age tend to produce female offspring with reduced fertility index.

The positive, non-significant, genetic correlations between GH, insulin and PLA_a suggests that higher concentrations of GH and insulin in 4 month old calves is associated with a higher than average proportion of samples with luteal activity within 60 days postpartum (*Figure 6.6*). Both insulin and GH play an important role in reproductive function with GH stimulating progesterone and oxytocin synthesis by the luteal cells (Schams & Berisha, 2004) and insulin stimulating steroid production by the growing follicle, granulosa cell proliferation, and it stimulates progesterone production by the corpus luteum (Langhout *et al.*, 1991; Mihm *et al.*, 2002; Webb *et al.*, 2004; See Chapter 1 for details). However, in Chapter 4 neither GH nor insulin were genetically correlated to the Danish fertility index but these (Chapter 4) were the concentrations following a fast in male calves.

The genetic correlations between PLA_w with glucose, FFA, GH, insulin and IGF-1 were the same direction and of a similar size, though not significant, to those with PLA_a (*Figure 6.6*). This is expected because the only difference between PLA_a and PLA_w is the frequency of sampling with PLA_a using three time a week milk samples and PLA_w using samples taken weekly from 0 – 60 days postpartum. The genetic correlations between PLA_f (fortnightly milk samples used) with FFA were negative and not significant as for PLA_a and PLA_w (*Figure 6.6*). Similarly the genetic correlation between PLA_f and insulin was positive but not significant again as for PLA_a and PLA_w. However, the genetic correlation between glucose and IGF-1 concentrations with PLA_f were positive and non-significant, unlike for PLA_a and PLA_w, this suggests that high concentrations of glucose and IGF-1 are associated with a high percentage of samples with luteal activity upto 60 days postpartum when sampling is fortnightly. This is perhaps what would have been expected for each of the PLA measures. As discussed previously glucose and IGF-1 are important in follicle growth and indeed are thought to link the nutritional status of an animal with reproduction, particularly during energy shortage (Butler, 2000; Roche *et al.*, 2000; Diskin *et al.*, 2003). However as this

measure is only based on fortnightly samples, which over 60 days will be between 4 and 5 samples, it is possible that the PLA_f figure may not be representative of the animals luteal activity due to the random nature of sample selection.

Finally, the genetic correlations of PLA_m with the hormone and metabolite concentrations were not comparable with the other PLA measures and furthermore the estimates were not significant and the standard errors were very high (0.66 to 1.93). The high standard errors, small dataset (n = 284 – 291) and infrequent sampling (monthly samples therefore 1-2 during 60 days postpartum) recommends that these results should not be interpreted and that genetic analysis of PLA_m should only be carried out with large datasets (n = >1000).

These analyses have shown that physiological fertility parameters CLA, PLA_a, PLA_w and PLA_f are not significantly genetically correlated to glucose, FFA, GH, insulin and IGF-1 concentrations in 4 month old female calves.

6.4.6 Approximate genetic correlations of hormones and metabolites with sire PTAs

Some of the approximate genetic correlations between glucose, FFA, GH, insulin and IGF-1 and the sire PTAs were moderate although mainly non-significant. Furthermore when interpreting approximate genetic correlations, calculated from regression coefficients, the estimate is largely dependent on the genetic standard deviations of the separate traits. This is particularly an issue for the sire PTAs as these genetic standard deviations were obtained from previous analyses but not from the actual analyses that produced the sire PTAs.

The approximate genetic correlation between GH concentration at 4 months of age with the sire PTA for calving interval was positive and significant (*Table 6.14*). This indicates that at a genetic level, high concentrations of GH are associated with a long calving interval. In agreement with the genetic correlation with calving interval, the

regression coefficient of fertility index on GH was significant and negative suggesting that high growth hormone is associated with a low fertility index (measures in pounds sterling; *Table 6.14*). Furthermore the genetic correlations between GH and PLA_a, although non-significant, indicate that high GH concentrations at four months of age are associated with a high proportion of samples with luteal activity. However, PLA is difficult to interpret as an intermediate PLA is probably most often associated with normal resumption of cyclicity (See Chapter 1.6). Perhaps the present approximate genetic correlation implies that high concentrations of GH at four months of age are associated with high concentrations of GH postpartum which is often seen during NEB and associated with poor resumption of ovarian cyclicity (Butler, 2000; Dechow *et al.*, 2002; de Vries & Veerkamp, 2000). However, it is actually the down-regulation of liver GH receptors that causes low IGF-1 concentrations (Radcliff *et al.*, 2003) and in part, reduced fertility in the postpartum period.

The approximate genetic correlation between protein percentage sire PTA and insulin was positive (0.82) whereas with FFA was negative (-0.87), both of which were significant (*Table 6.15 & 6.13*). Insulin, in response to high blood glucose concentrations, stimulates glycogen synthesis and protein synthesis whereas when blood glucose is low glucagon stimulates, amongst other things, the breakdown of stored fat which increases plasma FFA concentration (Jiang & Zhang, 2003). The direction of these relationships with protein percentage are logical, in that when blood glucose is high insulin will be released and precursors will be available for milk protein synthesis. However, when blood glucose is low, and consequently insulin is low, glucagon will increase FFA concentration and restrict protein synthesis (Jiang & Zhang, 2003). Of interest also, is the genetic correlation between insulin and CLA (*Table 6.6*) although non-significant was negative, suggesting that low plasma insulin is associated with long CLA. It is possible that low milk protein percentage, associated with low plasma insulin, may be genetically correlated with long CLA. Pryce *et al.* (2000) reported that milk protein yield was positively genetically correlated with calving interval i.e. low protein yield associated with short calving interval. However, protein percentage and protein

yield are not directly comparable; milk with identical protein yields can differ in protein percentage due to differences in the total volume of milk.

The approximate genetic correlations and regressions in the present study were mainly non-significant and as described, the approximate genetic correlations are largely dependent on the genetic standard deviations used in the calculation. Furthermore, the dataset here (n = 1077) and the number of animals with sire PTAs available (Milk production PTAs n = 1035; fertility PTAs n = 563 to 983) was small (Section 6.4.1) and therefore correlations, especially when not significant, should be interpreted with caution.

6.4.7 Future work

These analyses, described above, have not provided conclusive evidence as to whether glucose, FFA, GH, insulin and IGF-1 are or are not genetically and phenotypically correlated with physiological fertility parameters (CLA, PLA_a, PLA_w and PLA_f). Some of the approximate genetic correlations with sire PTAs for production and fertility were moderate and significant. However, the limiting factor in this analysis has been the relatively small dataset leading to insignificant correlations and large standard errors (Section 6.4.1). To obtain smaller standard errors and ascertain whether these genetic relationships are significantly different to zero a larger dataset is needed. Unfortunately due to the small number of males tested (n = 256) it was not possible to analyze the genetic correlation of the hormones and metabolites with the sire PTAs which may have provided important answers with regards to the possibility of using glucose, FFA, GH, insulin or IGF-1 as a juvenile predictor of fertility measured in the 4 month old male.

Chapter 7: GENERAL SUMMARY AND DISCUSSION

The main findings of this thesis are that free fatty acids (FFA), glucose, growth hormone (GH), insulin and insulin like growth factor 1 (IGF-1) concentrations are moderately heritable in pre-pubertal (9 months of age, Chapter 4; 4 months of age, Chapter 5) male and female dairy calves (Danish Holstein, Danish Jersey, Red Dane, Chapter 4; Holstein-Friesian, Chapter 5). These findings are largely supportive of previous findings in 9 month old calves of different breeds (Løvendahl *et al.*, 1994; Løvendahl and Jensen, 1997; Davis and Simmen, 2000; Grochowska *et al.*, 2001). In the Danish experiment (Chapter 4), male calves with low FFA and glucose concentrations at 9 months of age following a fast were genetically more likely to produce female offspring with a better fertility index than those with high FFA and glucose concentrations. Yet in the UK experiment (Chapter 5 & 6), fed calves with high glucose and insulin concentrations at 4 months of age tended to have a shorter interval to luteal activity as heifers. Although the Danish fertility index is not directly comparable with the interval to commencement of luteal activity postpartum (CLA) it is likely that the two measures are genetically related. The Danish fertility index (at the time of writing this thesis) combined information on days from first to last insemination in heifers and cows, and days from calving to first insemination in cows and since the latter of these has been shown to be genetically correlated with CLA (genetic correlation 0.53 $P<0.0005$, $n = 148$; Royal *et al.*, 2003) it is likely that some comparison between the Danish fertility index and CLA is reasonable although not ideal.

If the results presented in this thesis are confirmed, the possible application of this knowledge would not be straightforward. However with female fertility levels in the UK and in the worldwide dairy population being low (conception rate to first service 39.7%; Royal *et al.*, 2000a; 37%; Mayne *et al.*, 2002) and with other equally important concerns facing the dairy industry such as welfare, disease, and falling milk prices, the possibility of a method to increase the rate of genetic improvement in fertility should be considered. From this initial research it seems most probable that the concentration of glucose, FFA and insulin in blood plasma may be linked to future female fertility.

Important questions at this stage are at what age, in which sex and at what nutrition status (fed or fasted) should the animals be when measurements are taken. Furthermore, would it be feasible to use this method of assessment at the commercial dairy farm level or would it only be practical at a breeding company level. In addition, would blood samples need to be sent to a laboratory for analysis or could a simple kit be designed such as has been done for milk progesterone testing (Ridgeway Science Ltd., Alvington, Gloucestershire, UK). What would be the criteria or concentration for the test; should calves with the highest concentrations of glucose, FFA and insulin be selected for breeding or should the calves with the lowest concentrations be rejected.

In answer to the above, our results suggest that this measurement could be taken at 4-5 months of age or at 9 months of age. From a financial point of view the sooner a selection decision can be made the better because costs of rearing animals, that would later be rejected, could be reduced. Results from Chapter 5 suggest that the age of animal at testing (range, 72 – 167 d) had a significant effect on insulin and IGF-1 concentrations but not on glucose, FFA or GH. Concentrations of insulin and IGF-1 increase with age (birth until ≥15 months of age; Plouzek & Trenkle, 1991; Skaar *et al.*, 1994) whereas in contrast glucose (Rowlands *et al.*, 1983) and FFA (Quigley *et al.*, 1991) remain relatively stable over the range of ages at testing (range, 72 – 167 d) and vary due to feeding status. GH concentrations decrease with age (birth until ≥52 weeks of age; Govoni *et al.*, 2003). The age of animal at testing would therefore need to be constant and any deviation from this age would require a correction factor.

Any such test would need to be applicable in both sexes and most importantly in the male, since the male can produce thousands of offspring in his lifetime whereas a female will usually produce no more than five. Chapter 5 highlighted that the concentrations of the hormones studied were significantly affected ($P<0.001$) by the sex of calf sampled; with males tending to have increased concentrations (with the exception of insulin). Previous studies examining hormone concentrations in calves have also reported higher concentrations in males than females of the same age (Keller *et al.*, 1979; Plouzek & Trenkle, 1991; Govoni *et al.*, 2003). There is evidence to suggest that concentrations of

these hormones and metabolites are higher in male than in female calves though this does not mean that genetically they are different traits, it may be that males mature at a faster rate and therefore concentrations are higher at a younger age than in females. Additionally, the concentrations of glucose, FFA, insulin and GH have been found to be strongly genetically correlated between male and female calves at 9 months of age (genetic correlation range, 0.45 to 1.00; Løvendahl and Jensen, 1997) indicating that they are probably genetically the same trait. This highlights the need to adjust the criteria of the test according to the sex of the animal tested. From a management perspective it would be easier to carry out this test in fed animals; however the relationship between the fertility index or CLA with glucose is altered by whether the animal is fed or fasted (Chapter 4 & 6) therefore the feeding state would need to be consistent. The benefit of using fasted animals is that the time elapsed since last feeding is constant and therefore residual variation in the hormone concentration is reduced though at least 24 hours is needed to fully starve a calf and this imposes a time constraint and a welfare issue. Furthermore, results in Chapter 5 highlight the importance of following a strict experimental protocol prior to and during blood sampling to reduce variation in hormone concentrations due to "stress" caused by inadequate time left to acclimatize to the surroundings prior to blood sampling.

The measurement of the concentration of glucose, FFA and insulin in blood plasma of calves could be potentially used by both breeding companies and individual farms that breed their own replacement heifers. The practicality of this method would be determined, in part, by the cost of the test, the analysis i.e. laboratory or by on farm kit, the time taken to get the results, the time taken to carry out the test which would be extended if fasting was required, the certainty and risk of selection decisions and the predicted benefits. At this stage it is too earlier to consider using this test in calves. However as results accumulate in this research area in the future it would be advisable to limit the risk associated with selecting the wrong animals by imposing a low selection intensity for this test and by using this test to reject the "worst" calves rather than select the "best".

This is the first research at a genetic level into the potential use of metabolic hormones as juvenile predictors for fertility and inevitably further research is needed. The high standard errors and lack of significant genetic correlations between the hormones and metabolites with the physiological fertility measures (CLA and PLA) and the sire PTAs is possibly due to the small dataset (n = 440 CLA and PLA; n = 1035 Milk production PTAs; n = 563 to 983 fertility PTAs). Furthermore, while many relationships found appear interesting, the results should be interpreted with care. This research would have benefited from additional male calf data (only data on 256 males were analysed in Chapter 5 & 6), additional physiological (CLA and PLA) and traditional fertility parameters such as calving interval and conception rate to first service for the calves as heifers, and fertility information collected on the female offspring of the male calves in Dataset 1. Unfortunately, this was not possible during this study due links between Cogent Breeding and Grosvenor Farms Ltd., who were both jointly involved in the research, being severed and calf sampling stopping thereafter.

Research into the use of physiological juvenile indicator traits for fertility in dairy cattle is sparse. Previous research has examined luteinizing hormone (LH) and testosterone responses to gonadotrophin releasing hormone (GnRH) in cattle and sheep (e.g. Haley *et al.*, 1989; Haley *et al.*, 1990; Mackinnon *et al.*, 1991; Royal *et al.*, 2000). Metabolic hormones have been studied with a view to juvenile prediction of milk yield (reviewed by Woolliams and Løvendahl, 1991) but as yet the results are not clear nor in use. IGF-1 has been widely studied as a predictor of liveweight gain in pigs and cattle (e.g. Suzuki *et al.*, 2004; Davis & Simmen, 2006). Despite the research, juvenile predictors are not commonly used. Quantitative trait loci (QTL) for fertility, health and production are being sought in all livestock species and it would appear that genomics has more potential to improve the rates of genetic improvement possible. The refinement of dense marker maps for the bovine genome (The Bovine Genome Database, USDA NRI, the Kleberg Foundation and the Texas Agricultural Experiment Station, USA) will aid the identification and fine mapping of potential QTL. With a denser marker map and more precisely located QTL, marker assisted selection (MAS) has the potential to accelerate genetic improvement and reduce the inaccuracy associated with phenotypic selection

alone. However the value of MAS is dependent on many things such as the size of the QTL effect, the frequency of the positive QTL allele in the population and the extent to which the QTL and marker are linked. It is likely that in the future, a combination of the use of physiological measurements, such as hormone concentration in a blood sample, and MAS will be used for the prepubertal assessment of potential fertility in male and female calves.

The objective of this thesis was to ascertain the suitability of FFA, glucose, GH, insulin and IGF-1 as prepubertal indicators of female fertility. This research has indicated that an appropriate level of genetic variation is present in all traits investigated to potentially be useful for juvenile selection criterion provided this is following a strict experimental procedure for some hours before sampling and management practices within the population of animals used is stringent to avoid additional error variance. However, the genetic relationships with fertility and production need further investigation in a larger dataset to clarify direction and strength of the correlations. Furthermore, although results suggest that FFA, glucose and insulin concentrations in prepubertal calves are genetically correlated with female fertility the results are not conclusive (small correlations, large standard errors and non-significance). The protocol and whether fasted or fed appears to affect the direction of the genetic correlation and the magnitude of the residual variance. Further research is needed to confirm results found here, determine the correlated response to selection in other traits and to assess the practicality of using such measurements in breeding companies and dairy herds to make genetic improvements in female fertility. Nevertheless, the research described in this thesis although not conclusive has improved our understanding of the genetic variation and genetic relationships between metabolic hormones and fertility in dairy cattle.

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